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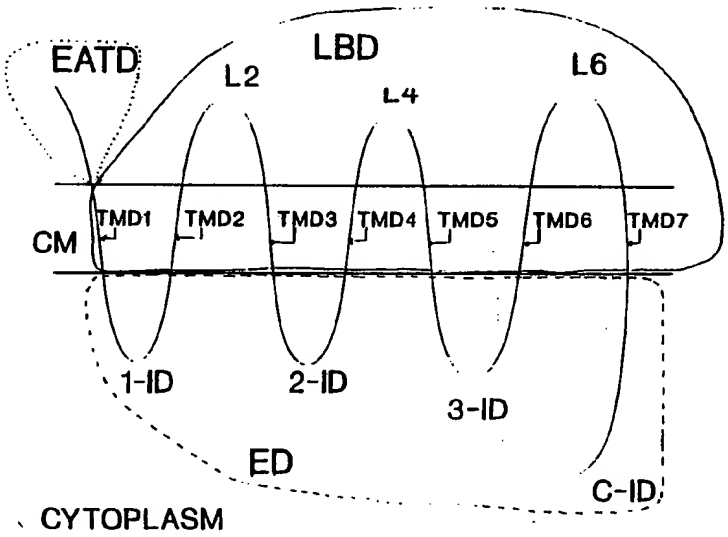

(57) Abstract

Methods are disclosed for producing hybrid G protein-coupled receptors. DNA sequences encoding hybrid G protein-coupled receptors are provided, wherein the receptors comprise mammalian G protein-coupled receptors having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor. DNA constructs comprising the following operatively linked elements: a transcriptional promoter, a DNA sequence encoding a hybrid G protein-coupled receptor, wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor, and a transcriptional terminator. Host cells transformed with the DNA constructs and methods utilizing the transformed cells are also provided.

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Description

METHODS OF PRODUCING HYBRID G PROTEIN-COUPLED RECEPTORS

Technical Field

The present invention is generally directed toward the expression of proteins, and more specifically, toward the expression of hybrid G protein-coupled receptors in yeast.

Background of the Invention

In higher eukaryotic cells, the interaction between ligands (e.g. hormones) and receptors is of central importance in the transmission of and response to extracellular signals. Numerous physiologically important substances elicit cellular responses by binding to and acting on cell surface receptors. Examples of such substances include epinephrine, norepinephrine, isoproterenol and acetylcholine. The ligand-receptor binding mechanism is coupled to an effector mechanism to provide an appropriate cellular response. These mechanisms are often, but not always, combined in a single protein which is integrated into the cell membrane.

One class of receptors requires the presence of proteins which are interposed between the ligand-receptor binding mechanism and the effector mechanism. Upon binding to ligand, receptors of this class interact with guanine nucleotide-binding regulatory proteins (referred to herein as G proteins) which facilitate the transmission of the ligand binding signal (for review see Gilman, Cell 36:577-579, 1984 and Biochemistry 26:2657-2664, 1987) from the cell surface to the specific cell mechanism(s) to be activated. This class of receptors is generally referred as G protein-coupled receptors.

G protein-coupled receptors mediate important physiological responses, which include vasodilation, stimulation or decrease in heart rate, bronchodilation,

stimulation of endocrine secretions and enhancement of gut peristalsis. One group of G protein-coupled receptors, the adrenergic receptors are found in a variety of higher eukaryotic tissues and mediate a diversity of physiological responses (for review see, Lefkowitz et al., Ann. Rev. Biochem. 52:159-186, 1983). Ahlquist (Am. J. Physiol. 153:586-600, 1948) proposed that adrenergic receptors fall into two classes, α and β, based on the order of activity of a series of ligands. Lands, (Nature 214:597-598,1964), Starke (Revs. Physiol. Biochem. Pharmacol. 77:1-124, 1977), and Langer et al. (Biochem. Pharmacol. 23:1793-1800, 1974) further divided these classes into α1, α2 and β1, β2. Lands (ibid.) designated β1 receptors as those β-adrenergic receptors (referred to herein as βARs) responsible for cardiac stimulation and lipolysis and β2 receptors as those βARs that mediate adrenergic bronchodilation and vasodepression. Ligands to βARs are used in the treatment of anaphylaxis, shock, hypotension, cardiogenic shock, asthma, premature labor, angina, hypertension, cardiac arrhythmias, migraine and hyperthyroidism.

While ligands to G protein-coupled receptors have potential as therapeutic agents, screening for these compounds is both difficult and labor intensive. Currently, ligand binding is measured using radioligand binding methods (Lefkowitz et al., Biochem. Biophys. Res. Commun. 60:703-709, 1974; Aurbach et al., Science 186:1223-1225, 1974; Atlas et al., Proc. Natl. Acad. Sci. USA 71:4246-4248, 1974). Potential agonists can be directly assayed using the radio-ligand binding methods by binding radiolabelled substances to a membrane fraction or to responsive cells. The amount of radioactivity remaining after the excess label is removed is the measure of substance bound to the receptors. Antagonists can be screened by their ability to compete with a known labeled agonist for cell surface receptors, thus reducing the amount of radioactivity bound to the membranes or cell

surfaces. In the case of βARs, this method first involves the isolation of intact membranes from responsive tissues or cell lines. Often, only a limited subset of cells is responsive to a particular agent (Lefkowitz et al., Ann. Rev. Biochem. 52:159-186, 1983) and such cells may be difficult to grow in culture or may possess a low number of receptors, making assays cumbersome. In addition, mammalian cells co-express a variety of G protein-coupled receptor classes and subclasses making ligand screening for any one particular class of receptors difficult. The current assay system is labor intensive and does not lend itself to automation and high through-put screening assays. The use of cultured mammalian tissues as a source of receptors is both difficult and expensive.

Although human βARs have been expressed in E. coli (Marullo et al., Proc. Natl. Acad. Sci. USA 85:7551-7555, 1988; and Marullo et al.,Bio/Technology 7:923-927, 1989), the level of receptor expression is very low and ligand binding assays are limited to the multiple-step, labor-intensive radioligand assay used for mammalian cells. As such, these transformed cells are not useful for commercial scale, high through-put ligand screening.

There is therefore a need in the art for an assay system which permits high volume screening of compounds which may act on higher eukaryotic cells via G protein-coupled receptors. Such a system should be rapid, inexpensive and adaptable to high volume screening. The present invention provides such an assay system and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention discloses DNA sequences encoding hybrid G protein-coupled receptors. These hybrid G protein-coupled receptors, when expressed in appropriate host cells, allow screening of potential ligands to mammalian G protein-coupled receptors using a standardized method. The invention also provides a

variety of methods for detecting the presence of ligand in a test substance all using a single cell type, thus providing for standardized detection methods not previously available in the art. The host cells of the present invention provide the further advantages of being easily cultured and respond to ligands in an easily monitored manner.

In one aspect of the invention DNA sequences encoding hybrid G protein-coupled receptor are disclosed wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor. In one embodiment of the invention, the yeast G protein-coupled receptor is selected from the group consisting of the Saccharomyces cerevisiae STE2 gene product, the Saccharomyces cerevisiae STE3 gene product and the Saccharomyces kluyveri STE2 gene product. In a preferred embodiment, the yeast G protein-coupled receptor is the Saccharomyces cerevisiae STE2 gene product. In another embodiment of the invention, the mammalian G protein-coupled receptor is selected from the group consisting of β-adrenergic receptors, α-adrenergic receptors, muscarinic receptors, angiotensin receptors, substance K receptors and rhodopsin receptors. In one embodiment, the DNA sequence encodes a hybrid mammalian G protein-coupled receptor wherein the mammalian G protein-coupled receptor domain selected from the group consisting of at least a portion of the extracellular amino-terminal domain, the effector domain, the third internal effector domain and the carboxy-terminal internal effector domain is replaced with the corresponding domain of a yeast G protein-coupled receptor. In another embodiment of the invention, the DNA sequence encodes a hybrid mammalian G protein-coupled receptor wherein the mammalian G protein-coupled receptor domains selected from the group consisting of the extracellular amino-terminal and effector domains of the mammalian G protein-coupled

receptor are replaced with the extracellular amino-terminal and effector domains of a yeast G protein-coupled receptor. In yet another embodiment, the DNA sequence encodes a hybrid mammalian G protein-coupled receptor wherein the mammalian G protein-coupled receptor domains selected from the group consisting of the carboxy-terminal internal effector domain, the third internal effector domain, and the carboxy-terminal internal effector and third internal effector domains are replaced by the corresponding domains of a yeast G protein-coupled receptor.

Another aspect of the invention is directed towards a DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor in a yeast cell, comprising the following operatively linked elements: a transcriptional promoter; a DNA sequence encoding a biologically active hybrid G protein-coupled receptor, said receptor comprising a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with the corresponding domain of a yeast G protein-coupled receptor; and a transcriptional terminator.

In a related aspect, the present invention discloses yeast host cells transformed with a DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor, said receptor comprising a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with the corresponding domain of a yeast G protein-coupled receptor. In a preferred embodiment, the yeast host cell is a Saccharomyces cerevisiae cell. In a particularly preferred embodiment, the yeast host cell is a Saccharomyces cerevisiae a haploid cell that does not contain a functional BAR1 gene. In another aspect of the invention, the yeast host cell is transformed with a second DNA construct comprising the BAR1 promoter operatively linked to an indicator DNA

sequence, and wherein the second DNA construct is integrated at the BAR1 locus. In a preferred embodiment, the indicator DNA sequence is the lacZ coding sequence.

The present invention discloses methods for detecting the presence of ligand in a test substance. The methods comprise the steps of a) exposing a culture of yeast host cells transformed with a DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor, and wherein said yeast host cells express the biologically active hybrid G protein-coupled receptor, to a test sample under suitable conditions to allow binding of ligand to the hybrid G protein-coupled receptor; and b) detecting a biological response of the host cell and therefrom determining the presence of the ligand. In one embodiment of the invention, the host cells are also transformed with a second DNA construct comprising the BAR1 promoter operatively linked to an indicator DNA sequence and the step of detecting comprises detecting the expression of said indicator DNA sequence. In a preferred embodiment, the method further comprises host cells that are Saccharomyces cerevisiae a haploid cells transformed with a second DNA construct comprising the BAR1 promoter operatively linked to the E. coli lacZ coding sequence wherein the second DNA construct is integrated at the BAR1 locus. In one embodiment of the invention, the method further comprises host cells that are suspended in an agar overlay on top of an appropriate solid growth medium. In related aspect of the invention, the agar overlay includes one or more wells and the step of exposing comprises filling the wells with the test substance. In another embodiment of the invention, the step of exposing comprises placing a filter saturated with the test substance onto the agar overlay. In one preferred embodiment, the method comprises host cells that are

Saccharomyces cerevisiae mating-type a haploid cell transformed with a DNA construct capable of directing the expression of a hybrid G protein-coupled receptor, wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a STE2 gene product, and wherein the step of detecting comprises detecting the presence of a halo of host cells arrested in the G1 phase of cell division. In another embodiment of the invention, the method comprises a culture of host cells suspended with an agonist in an agar overlay on top of an appropriate solid growth medium. In preferred embodiment of the invention, the method comprises Saccharomyces cerevisiae mating-type a host cells transformed with a DNA construct capable of directing the expression of a hybrid G protein-coupled receptor, wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a STE2 gene product, suspended with an agonist in an agar overlay on top of an appropriate solid growth medium, and wherein the step of detecting comprises detecting the presence of a halo of host cell colonies.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 illustrates the structure of a representative G protein-coupled receptor. Symbols used are EATD, which is encircled by the dotted line, extracellular amino-terminal domain; LBD, which is encircled by the solid line, the ligand-binding domain; ED, which is encircled by the dashed line, the effector domain; 1-ID, the first internal effector domain; 2-ID, the second internal effector domain; 3-ID, the third

internal effector domain; C-ID, the carboxy-terminal internal effector domain; L2, the first external ligand-binding domain; L4, the second external ligand-binding domain; L6, the third external ligand-binding domain; TMD1, the first transmembrane domain; TMD2, the second transmembrane domain; TMD3, the third transmembrane domain; TMD4, the fourth transmembrane domain; TMD5, the fifth transmembrane domain; TMD6, the sixth transmembrane domain, and TMD7, the seventh transmembrane domain.

Figure 2 illustrates a partial restriction map of representative STE2 clones pAH1, pAH2, pAH3 and STE2-SubP #6. Symbols used are B, Bam HI; E, Eco RI; H, Hind III; P, Pst I; Pv, Pvu II; S, Sal I; X, Xba I; subP, substance P. Open boxes indicates vector sequences, the hatched box refers to M13mp8 vector sequences.

Figure 3 illustrates a nucleotide sequence encoding a representative hamster G protein-coupled receptor, the hamster β_2AR and the inferred amino acid sequence of the protein. Numbers above the line refer to the nucleotide sequence of the mature protein. Boxed sequences refer to the second and third external ligand-binding domains. Symbols L2 and L4 refer to the first, second and third external ligand-binding domains, respectively.

Figure 4 illustrates the construction of plasmid pHRS6. Symbols used are as in Figure 1, and STE2, Saccharomyces cerevisiae STE2 genomic sequence.

Figure 5 illustrates the contruction of plasmid pHRS5. Symbols used are as in Figure 1, and STE2, Saccharomyces cerevisiae STE2 genomic sequence; subP, substance P C-terminal pentapeptide dimer coding sequence.

Figure 6 illustrates the construction of plasmid pHRS9. Symbols used are as in Figure 1, and STE2, Saccharomyces cerevisiae STE2 genomic sequence; subP, substance P C-terminal pentapeptide dimer coding sequence.

Figure 7 illustrates a nucleotide sequence encoding a representative human G protein-coupled

receptor, the human β_2AR and the inferred amino acid sequence of the protein. Numbers above the line refer to the nucleotide sequence of the mature protein. Solid lines above the sequence refer to the putative transmembrane domains. Symbols used are as for Figure 1.

Figure 8 illustrates the construction of plasmid pHRS11.

Figure 9 illustrates a nucleotide sequence encoding a representative yeast G protein-coupled receptor, the Saccharomyces cerevisiae STE2 gene and the inferred amino acid sequence of the protein. Numbers above the line refer to the nucletide sequence of the mature protein. Solid lines above the sequence refer to the putative transmembrane domains. Symbols used are as for Figure 1.

Figure 10 illustrates representative competitive binding curves for epinephrine and norepinephrine.

Figure 11 illustrates a representative competetive binding curve for isoproterenol.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Biological activity: A function or set of activities performed by a molecule in a biological context (i.e., in an organism or an in vitro facsimile thereof). Biological activities may include the induction of extracellular matrix secretion from responsive cell lines, the induction of hormone secretion, the induction of chemotaxis, the induction of differentiation, or the inhibition of cell division of responsive cells. A recombinant protein is considered to be biologically active if it exhibits one or more biological activities of its native counterpart.

A receptor is considered to be biologically active if it is capable of binding ligand, transmitting a

signal and eliciting a cellular response. A yeast-expressed mammalian hybrid G protein-coupled receptor having a domain other than the ligand-binding domain replaced with a corresponding domain of a yeast pheromone receptor, for example, is biologically active if it is capable of binding ligand and inducing the mating response pathway, resulting in the G1 arrest of the yeast host cells.

Ligand: A molecule capable of being bound by the ligand-binding domain of a receptor. The molecule may be chemically synthesized or may occur in nature.

Domain: A portion of a protein or peptide that is physically or functionally distinguished from other portions of the protein or peptide. Physically-defined domains include those amino acid sequences that are exceptionally hydrophobic or hydrophilic, such as those sequences that are membrane-associated or cytoplasm-associated. Domains may also be defined by internal homologies that arise, for example, from gene duplication. Functionally-defined domains have a distinct biological function(s). The ligand-binding domain of a receptor, for example, is that domain that binds ligand. Functionally-defined domains need not be encoded by contiguous amino acid sequences. Functionally-defined domains may contain one or more physically-defined domain. Receptors, for example, are generally divided into a ligand-binding domain and an effector domain. G protein-coupled receptors are generally divided into an extracellular amino-terminal domain, a ligand-binding domain, and an effector domain.

As noted above, a variety of physiological responses of higher eukaryotic cells are mediated by G protein-coupled receptors. Ligands to these receptors are used to treat a variety of conditions. Currently available methods for screening potential G protein-coupled receptor ligands are expensive, labor intensive

and are limited by the necessity of isolating membrane fragments from responsive tissues or cell lines.

The present invention provides hybrid G protein-coupled receptors. These hybrid receptors comprise a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor. The invention further provides DNA constructs capable of directing the expression of such DNA sequences, eukaryotic cells transformed with such DNA constructs, and methods for assaying ligand binding using such cells. The invention thus provides cross-species hybrid G protein-coupled receptors not previously known.

While not wishing to be bound by a graphical representation, G protein-coupled receptors are believed to have the general structure shown in Figure 1. These receptors comprise an extracellular amino-terminal domain, a ligand-binding domain and an effector domain (Figure 1). Comparisons of avian and mammalian β-adrenergic receptor cDNA's (Yarden et al., Proc. Natl. Acad. Sci. USA 83:6795-6799, 1986; Dixon et al., Nature 321:75-79, 1986; and Kobilka et al., Proc. Natl. Acad. Sci. USA 84:46-50, 1987), a bovine rhodopsin cDNA (Nathans and Hogness, Cell 34:807-814, 1983), an α_2-adrenergic receptor (Kobilka et al., Science 238:650-656, 1987), an angiotensin receptor cDNA (Young et al., Cell 45: 711-719, 1986; Jackson et al., Nature 335:437-439, 1988), a bovine substance K receptor (Masu et al., Nature 329:836-838, 1987), and a muscarinic acetylcholine receptor cDNA (Kubo et al., Nature 323:411-416, 1986) predict that all six proteins share the structure shown in Figure 1 (for review see Lefkowitz et al., J. Biol. Chem. 263:4993-4996, 1988; Panayotou and Waterfield, Curr Opinion Cell Biol. 1:167-176, 1989).

As used herein, the ligand-binding domain of a G protein-coupled receptor is that portion of the receptor, shown in Figure 1 as LBD, that is involved in binding

ligand and generally comprises that portion of the receptor containing the transmembrane domains (TMDs) and their associated extracellular ligand-binding domains. The structure of G protein-coupled receptors may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mt. View, CA) or may be predicted according to the methods described, for example, by Kyte and Doolittle, J. Mol. Biol. 157:105-132, 1982). The ligand-binding domain of the β_2-adrenergic receptor, for example, has been shown to require at least the third, fifth, and seventh transmembrane domains (Dixon et al., Nature 326:73-77, 1987; Strader et al., J. Biol. Chem. 263:10267-10271, 1988; Strader et al., J. Biol. Chem. 264:13572-13578, 1989). The effector domain of a G protein-coupled receptor, shown in Figure 1 as ED, is that domain of a G protein-coupled receptor that may be phosphorylated and may be involved in the interaction with associated G proteins and in the mechanisms of desensitization, adaptation, internalization and recycling of the receptor-ligand complex. The effector domain is understood to be encoded by amino acid sequences that need not be contiguous and may include the first, second, third and/or carboxy-terminal internal effector domains (Figure 1 as 1-ID, 2-ID, 3-ID and C-ID, respectively). Dixon et al. (ibid., 1987), for example, have suggested that the effector domain of a human β_2AR includes the third internal domain.

The present invention makes use of the ability of eukaryotic cells to respond to stimuli via G protein-coupled receptors. In one embodiment of the invention, for example, DNA sequences encoding hybrid G protein-coupled receptors, when expressed in yeast host cells, enable the host cells to bind and respond, through a yeast biological response, to G protein-coupled receptor ligands that would not otherwise elicit a such a response. A representative such response is that of yeast cells to

mating pheromones. Cells of the yeasts Saccharomyces cerevisiae and Saccharomyces kluyveri are responsive to the external mating pheromones α-factor and a-factor. Saccharomyces cerevisiae and Saccaromyces kluyveri MATa cells express STE2 gene products that have have been shown to be the α-factor receptor (Jenness et al., Cell 35:521-529, 1983; Nakayama et al., EMBO J. 4:2643-2648, 1985; Burkholder and Hartwell, Nuc. Acids Res. 13:8463-8475, 1985; Marsh and Herskowitz, Proc. Natl. Acad. Sci. USA 85:3855-3859, 1988). Saccharomyces cerevisiae MATα cells, express the STE3 gene product which has been shown to be the a-factor receptor (Nakayama et al., EMBO J. 4:2643-2648, 1985; Hagen et al., Proc. Natl. Acad. Sci. USA 83:1418-1422, 1986). Although the mechanism(s) by which these putative receptors mediate cellular responses has not been elucidated, it is generally believed that these receptors are coupled to G-proteins (Whiteway et al., Cell 56:467-477, 1989; Herskowitz and Marsh, Cell 50: 995-996, 1987). The binding of mating pheromones to their respective receptors activates the mating pheromone response pathway. The response pathway is believed to be mediated, in part, by the SCG1, STE4 and STE18 gene products and leads to the transcriptional induction of mating-type specific genes and agglutinin genes, and to the arrest of cells in the G1 phase of cell division. The present invention utilizes DNA sequences encoding hybrid G protein-coupled receptors that, when expressed by yeast host cells, enable the host cells to bind and respond to G protein-coupled receptor ligands that would not otherwise elicit a yeast mating response.

DNA sequences encoding hybrid G protein-coupled receptors may be prepared from cloned receptor DNAs using standard techniques of restriction enzyme digestion, exonuclease digestion and ligation or may be prepared by *in vitro* mutagenesis using, for example, the method described by Zoller and Smith (DNA 3:479-488, 1984) or Kunkel (Proc. Natl. Acad. Sci. USA 82:488-492, 1985) to

replace the DNA sequence encoding at least one domain, other than the ligand-binding domain, of a mammalian G protein-coupled receptor with the DNA sequence encoding the corresponding domain of a yeast G protein-coupled receptor. One exemplary DNA sequence encoding a hybrid G protein-coupled receptor encodes a hybrid human βAR wherein the amino-terminal extracellular domain is replaced with the amino-terminal extracellular domain of the Saccharomyces cerevisiae STE2 gene product. Another exemplary DNA sequence encoding a hybrid G protein-coupled receptor encodes a hybrid human βAR wherein the carboxy-terminal internal effector domain is replaced with the carboxy-terminal internal effector domain of the Saccharomyces cerevisiae STE2 gene product. Another exemplary DNA sequence encoding a hybrid G protein-coupled receptor encodes a hybrid human βAR wherein the amino-terminal extracellular and carboxy-terminal internal effector domains are replaced with the amino-terminal extracellular and carboxy-terminal internal effector domains of the Saccharomyces cerevisiae STE2 gene product. Another exemplary DNA sequence encoding a hybrid G protein-coupled receptor encodes a hybrid human βAR wherein the amino-terminal extracellular domain, the third internal effector domain and carboxy-terminal internal effector domain are replaced with the amino-terminal extracellular domain, the third internal effector domain and carboxy-terminal internal effector domain of the Saccharomyces cerevisiae STE2 gene product.

Complementary DNAs encoding a human β_2AR (Kobilka et al., ibid.), a human β_1AR (Frielle et al., Proc. Natl. Acad. Sci. USA 84:7920-7924, 1987), a hamster β_2AR (Dixon et al., ibid., 1986), a turkey βAR (Yarden et al., ibid.), a rhodopsin receptor (Nathands and Hogness, ibid.), an a_2-adrenergic receptor (Kobilka et al., ibid., 1987), an angiotensin receptor (Young et al., ibid.; Jackson et al., ibid.), a substance K receptor (Masu et al., ibid.), and a muscarinic acetylcholine receptor (Kubo

et al., ibid.) have been described. Alternatively, these and other G protein-coupled receptor DNAs may be cloned from cDNA libraries prepared from appropriate cell lines and isolated by homology to cloned genomic or cDNA sequences encoding G protein-coupled receptors or using antibodies directed against the receptor. Alternatively, cDNA libraries may be constructed into expression vectors and G-protein-coupled receptor DNAs may be isolated by the identification of cells expressing the G protein-coupled receptor. DNA sequences encoding mammalian G protein-coupled receptors may also be synthesized using standard techniques. In general, cDNA sequences are preferred for carrying out the present invention due to their lack of intervening sequences which can lead to aberrant RNA processing and reduced expression levels, particularly in yeast cells. Complementary DNAs encoding a β_2AR, for example, may be obtained from libraries prepared from placental cells according to standard laboratory procedures and screened using genomic or cDNA sequences of known β_2ARs. If partial clones are obtained, it is necessary to join them in proper reading frame to produce a full length clone, using such techniques as endonuclease cleavage, ligation, and loop-out mutagenesis.

DNA sequences encoding yeast G protein-coupled receptors also have been described. For example, the Saccharomyces cerevisiae STE2 gene (Nakayama et al., EMBO J. 4:2643-2648, 1985; Burkholder and Hartwell, Nuc. Acids Res. 13:8463-8475, 1985), the Saccharomyces cerevisiae STE3 gene (Nakayama et al., EMBO J. 4:2643-2648, 1985; Hagen et al., Proc. Natl. Acad. Sci. USA 83:1418-1422, 1986 and Hagen et al., Proc. Natl. Acad. Sci. USA 83:1418-1422, 1986) and the Saccharomyces kluyveri STE2 gene (Marsh and Herskowitz, Proc. Natl. Acad. Sci. USA 85:3855-3859, 1988) have been described. DNA sequences encoding yeast G protein-coupled receptors may be cloned from DNA libraries prepared from yeast strains using the standard yeast techniques of transformation and complementation.

The Saccharomyces cerevisiae STE2 gene, for example, may be cloned using a DNA library prepared from wild type yeast cells to transform a Saccharomyces cerevisiae strain carrying a ste2 mutation. DNA sequences capable of complementing the ste2 mutation will enable the yeast host cells to mate.

DNA sequences encoding the hybrid receptor fusions are placed in suitable expression vectors for expression eukaryotic cells such as in yeast. Suitable yeast expression vectors include YRp7 (Struhl et al., Proc. Natl. Acad. Sci.USA 76:1035-1039), YEp13 (Broach et al. Gene 8:121-133, 1979), pJDB248 and pJDB219 (Beggs, ibid.) and derivatives thereof. Such vectors will generally include a selectable marker, such as the nutritional marker LEU2, which allows selection in a yeast host strain carrying a leu2 mutation. Another selectable marker that may be used is the POT1 gene described by Kawasaki and Bell (EP 171,141) that allows complementation of tpi1 mutations which render the host cell unable to grow in the presence of glucose.

Preferred promoters in yeast expression vectors include promoters from the Saccharomyces cerevisiae STE2 and STE3 genes (Hartig et al., Mol. Cell. Biol. 6:2106-2114, 1986; Nakayama et al., ibid.), Saccharomyces cerevisiae glycolytic genes (Hitzeman et al. J. Biol. Chem. 255:12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1:419-434, 1982) or Saccharomyces cerevisiae alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds), p. 335, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101:192-201, 1983). A particularly preferred promoter is the Saccharomyces cerevisiae TPI1 promoter (Alber and Kawasaki, ibid.; Kawasaki, U.S. Patent No. 4,599,311). In addition, it is preferrable to include a transcriptional termination signal, such as the TPI1 terminator, within the expression vector.

A number of eukaryotic cells may be used in the present invention. Preferred eukaryotic host cells for use in carrying out the present invention are strains of the yeast. Techniques for transforming yeast are well known in the literature, and have been described, for instance, by Beggs (Nature 275:104-108, 1978) and Hinnen et al. (Proc. Natl. Acad. Sci. USA 75: 1929-1933, 1984). Particularly preferred yeast host cells for use in the present invention are strains of Saccharomyces cerevisiae. In one embodiment of the invention, Saccharomyces cerevisiae cells that are MATa and do not produce a functional STE2 gene product are used as host cells. In a preferred embodiment, the Saccharomyces cerevisiae host cells are MATa cells containing a deletion of some or all of the STE2 gene. In another embodiment of the invention, the Saccharomyces cerevisiae host cells are MATa cells containing a genetic deficiency in the BAR1 gene. In a preferred embodiment of the invention, the Saccharomyces cerevisiae host cell are MATa cells containing a deletion of the BAR1 gene. In a particularly preferred embodiment of the invention the Saccharomyces cerevisiae host cells are MATa cells containing a deletion of the STE2 gene and a deletion of the BAR1 gene wherein the E. coli lacZ gene operatively linked to the BAR1 promoter replaces some or all of the BAR1 coding region. Suitable host strains may be obtained from depositories such as American Type Culture Collection, Rockville, Maryland, and the Yeast Genetic Stock Center, Berkeley, California, or may be prepared using standard mutagenesis techniques. Yeast host strains containing gene disruptions may be prepared, for example, by the method essentially described by Rothstein (Meth. Enzymology 101:202-211, 1983).

Transformed yeast host cells are obtained by selecting for the presence of the selectable marker. In general, selection of transformed cells is accomplished by complementation of the host's genetic defect by the selectable marker present on the plasmid. Yeast host

cells that are genetically leu2 and are transformed with vectors carrying the LEU2 marker, for example, are generally grown in a selective medium lacking the amino acid leucine.

After selection, the cells are grown in an appropriate growth medium to begin expressing the gene of interest. As used herein, the term "appropriate growth medium" means a medium containing nutrients and other components required for the selection and growth of transformed cells, and the expression of the DNA sequences encoding the hybrid G protein-coupled receptor. Media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins and salts. Media requirements will vary somewhat for particular host strains. Selection of an appropriate growth medium is within the level of ordinary skill in the art. In one embodiment, the medium is supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and the pH of the medium is preferrably maintained at a pH greater than 6.8 and less than 7.0. A stable pH may be maintained by buffering the medium. Suitable buffering agents including succinic acid, Bis-Tris (Sigma Chemical Co., St. Louis, MO) and potassium phosphate. The X-gal is preferrably supplemented at a concentration of 40 μg/ml. In some cases, solid growth medium may be required. An appropriate solid growth medium may be prepared for any appropriate growth medium by supplementing the media with between 1% and 3% agar, preferably 2% agar. Solid growth media is generally prepared by adding the agar to the growth medium prior to heat sterilization. Alternatively, a solid growth medium may be prepared by adding molten agar to sterile growth media.

Yeast host cells transformed with DNA constructs comprising DNA sequences encoding hybrid G protein-coupled receptors may be used in a variety of methods for detecting the presence of ligand in a test substance. These assays will generally include the steps of (a)

exposing a culture of yeast host cells transformed with a DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor, wherein the receptor is a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor, and wherein the yeast host cells express the biologically active hybrid G protein-coupled receptor, to the test sample under suitable conditions to allow binding of ligand to the hybrid G protein-coupled receptor and (b) detecting a biological response of the host cell and therefrom determining the presence of the ligand, wherein measuring is a means of detecting.

Suitable conditions to allow binding of ligand to a receptor are physiological conditions wherein the pH is maintained between 6 and 8, and the temperature is between 20°C and 40°C. Preferably the pH is maintained between pH 7.4 and 7.5 and the temperature is between 22°C and 23°C. As used herein, the binding of ligand to a receptor is understood to denote an interaction of a molecule with the ligand-binding domain of a receptor, which may result in a conformational change in the topology of the receptor. The binding of ligand to a receptor may either trigger or block a detectable biological response. Suitable biological responses for use in the present invention include the ability to mate, production of agglutinins, and adenylate cyclase activation. A particularly preferred biological response is cell division arrest in the G1 phase of cell division.

In one embodiment, the method comprises a culture of yeast cells transformed with a DNA construct capable directing the expression of a hybrid G protein-coupled receptor, wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled

receptor, is suspended in an agar overlay on top an appropriate solid growth medium. The agar overlay is preferably between 0.6% and 2.5% agar, preferably 0.7% agar. The agar may or may not be diluted in an appropriate solid growth medium. A solution containing the test substance is added to wells in the assay plate. Alternatively, filters saturated with the test substance are laid on the surface of the agar overlay. The test substance diffuses through the agar overlay and binds to the hybrid G protein-coupled receptors, inducing a biological reponse. A halo of responding cells indicates that the test substance contains an agonist.

Antagonists are detected by their ability to reverse or prevent the G1 arrest of cells that have been treated with a known agonist. In one the method, a culture host cells transformed with a DNA construct capable of directing the expression of a hybrid G protein-coupled receptor, the receptor comprising a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor, and wherein the yeast host cells express the hybrid G protein-coupled receptor, is suspended with an agonist in an agar overlay on top of an appropriate solid growth medium. The agonist induces a biological response of the host cells. A test substance is placed into wells in the assay plate or is saturated onto a filter that is laid on top of the agar. The test substance is allowed to diffuse through the media and competes with the agonist for binding to the hybrid G protein-coupled receptor. A halo of cells that have a reduced biological response colonies that the test substance contains an antagonist. In an alternate method, a culture yeast host cells transformed with a DNA construct capable of directing the expression of a hybrid G protein-coupled receptor, wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-

binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor, and wherein the yeast host cells express the hybrid G protein-coupled receptor, are suspended in an agar overlay of on top of an appropriate solid growth media. A test substance is mixed with an agonist and is placed into wells in the assay plate or is saturated onto a filter that is laid on top of the agar overlay. The test substance diffuses through the media and the test substance competes with the agonist for binding to the hybrid G protein-coupled receptors. A halo of cells exhibiting a reduced biological response relative to the biological response of host cells exposed to the agonist alone indicates that the test substance contains an antagonist.

Within preferred embodiment, the presence of a ligand in a test substance is detected on the basis of the ability of agonists to induce the yeast mating response pathway or antagonists to compete with agonists for binding with the receptor. In a particularly preferred embodiment the method comprises Saccharomyces cerevisiae host cells transformed with a DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor, wherein the receptor comprising a mammalian G protein-coupled receptor having at least one domain other than the the ligand-binding domain replaced with a corresponding domain of a yeast G protein-coupled receptor, and wherein the yeast host cells express the biologically active hybrid G protein-coupled receptor, is also transformed with a second DNA construct comprising a mating-type specific promoter operatively linked to an indicator DNA sequence. Within this method, the host cells are exposed to a test ligand under suitable conditions to allow binding of ligand to the hybrid G protein-coupled receptors, and binding of ligand to the receptors is detected by detecting the expression of the indicator DNA sequence. Mating-type specific gene promoters include promoters of the Saccharomyces

cerevisiae BAR1 gene, the Saccharomyces cerevisiae MFα1 gene, the Saccharomyces cerevisiae MFa1 gene, the Saccharomyces cerevisiae STE3 gene, the Saccharomyces cerevisiae STE2 gene, the Saccharomyces kluveyri gene, the Saccharomyces cerevisiae AGα1 gene, the Saccharomyces cerevisiae SST2 gene and the Saccharomyces cerevisiae FUS1 gene. A particularly preferred mating-type specific promoter for use in the present invention is the BAR1 promoter. Indicator DNA sequences include those DNA sequences whose expression results in a detectable biological response by the host cells. Suitable indicator DNA sequences include DNA sequences encoding nutritional markers that complement an auxotrophic host cell, DNA sequences that encode antibiotic resistance, and DNA sequences encoding enzymes capable of cleaving chromogenic substrates. A particularly preferred DNA sequence is the E. coli lacZ gene.

In a particularly preferred embodiment, the BAR1 promoter is operatively linked to the E. coli lacZ gene. The DNA construct is preferrably integrated at the BAR1 locus in the yeast genome, resulting in a substitution of the DNA construct for some or all of the endogenous BAR1 coding sequence.

In a particularly preferred embodiment of the invention, a method for detecting the presence of ligand in a test substance utilizes a culture of Saccharomyces cerevisiae mating-type a haploid host cells transformed with a DNA construct capable of directing the expression of a hybrid G protein-coupled receptor, wherein the receptor comprises a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with a corresponding domain of a STE2 gene product, and wherein the yeast host cells are transformed with a second DNA construct comprising the BAR1 promoter operatively linked to the E. coli lacZ coding sequence such that the second DNA construct is integrated at the BAR1 locus resulting in the substitution of into the host

cell genome part or all of the BAR1 sequence. The method comprises the steps of (a) exposing the culture of transformed host cells to a test substance under suitable conditions to allow ligand to bind to the hybrid G protein-coupled receptor and detecting the induction of the BAR1 promoter by measuring the level of β-galactosidase produced. In one embodiment of the invention, β-galactosidase expression is detected by measuring the production of the yellow cleavage product *o*-nitrophenol resulting from the cleavage of the chromogenic substrate *o*-nitrophenyl-β-D-galactoside with the β-galactosidase in host cell lysates. In another embodiment the host cells may be suspended as a lawn in top agar and poured over a plate of the medium comprising an appropriate growth media that has been buffered between pH 6.8 and pH 7.0 and supplemented with X-gal. The medium may be buffered with A solution containing the test substance is added to the wells in the assay plate, or test substance-saturated filters are laid on the surface of the agar overlay. The test substance diffuses through the soft agar overlay and binds to the hybrid G protein-coupled receptors, causing an induction of β-galactosidase expression. Ligand binding is detected by identifying the halos of blue cells, which result from the production of the deep blue dibromodichloroindigo produced from the cleavage of X-gal by the β-galactosidase. Blue colonies indicate that the test substance is an effective agonist.

The following examples are offered by way of illustration and not by limitation.

EXPERIMENTAL

EXAMPLE 1 - Cloning of the Saccharomyces cerevisiae STE2 gene

The STE2 gene was obtained as described by Hartig (Mol. Cell. Biol. 6:2106-2114, 1986). Briefly, a DNA library containing total yeast genomic fragments in

the vector YEp13, prepared as described by Nasmyth and Tatchell (Cell 19:753-764, 1980), was transformed into two leu2 yeast strains, each of which contained a ste2 mutation and was unable to mate. Transformed cells were isolated by selection on synthetic complete media lacking leucine. The Leu^+ colonies were screened for the ability to mate. Six colonies were identified that had acquired the ability to mate. Of the six colonies, five were found to contain different plasmids capable of complementing the ste2 mutations. The common region, found to be 2.6 kb in length, was demonstrated in plasmids pAH1 and pAH3 (Figure 2). The 2.6 kb Pst I-Bam HI fragment from pAH1 was subcloned into the yeast vector pZUC12 (obtained from Mogens Hansen, Novo-Nordisk A/S, Bagsvaerd, Denmark), which comprises the Saccharomyces cerevisiae LEU2 gene and the origin of replication from the Saccharomyces cerevisiae 2μm plasmid in the E. coli plasmid pUC12. Saccharomyces cerevisiae ste2 host cells transformed with the resultant plasmid were found to be capable of mating, confirming that the 2.6 kb insert from pAH1 contained the STE2 structural gene. The identity of the cloned gene was further confirmed by integration into the host genome and subsequent Southern hybridization. The approximately 2 kb fragment of plasmid pAH1 was subsequently sequenced and was found to contain the 1.2 kb STE2 coding region and associated 5′ flanking sequence. The DNA sequence of STE2 is shown in Figure 9.

The STE2 coding sequence present in pAH1 was subcloned into plasmid subPdimer-mp8 (Munro and Pelham, EMBO J. 3:3087-3093), which had been linearized with Sal I, to create plasmid STE2-SubP #6 (Figure 2). This truncated STE2-substance P fusion, upon subcloning into the yeast vector YEp13 and transformation into ste2 mutant, was shown to encode a protein which is capable of complementing the ste2 mutation in the host cell, allowing the cells to respond to α-factor and mate with MATa cells.

Example 2 - Expression of a Hamster β_2-Adrenergic Receptor-STE2 Fusions in Yeast Cells

A. Construction of DNA Constructs Encoding Hamster β_2-adrenergic receptor-STE2 Receptor Fusions

A hamster β_2AR (Dixon et al. ibid., 1986) and the Saccharomyces cerevisiae STE2 gene product have been predicted to share the structure shown in Figure 1. To study the relationship of the domains L2, L4 and L6 to ligand binding, the L2 and/or L4 domains of the STE2 gene product were replaced with the corresponding domains of the hamster β_2AR using in vitro mutagenesis (Zoller and Smith DNA 3:479-488, 1984) and linker addition.

The replacement of the STE2 L4 by the hamster β_2AR L4 was achieved by replacing the STE2 L4 with oligonucleotide adapters encoding the hamster β_2AR (Figure 3). Four oligonucleotides were designed to encode, upon annealing, a 5′ Hha I adhesive end followed by nucleotides 554 to 573 of Figure 8 encoding a portion of the STE2 TMD4 joined to a yeast codon-optimized hamster βAR L4 DNA sequence corresponding to nucleotides 522 to 585 of Figure 3 followed by an Nsi I adhesive end. Referring to Figure 4, plasmid pAH1 was cut with Sal I and Hha I to isolate the 1.3 kb fragment containing the partial coding region of STE2. Plasmid pAH1 was linearized with Sph I and partially cut with Nsi I to isolate the 0.8 kb fragment containing the STE2 sequences 3′ to the STE2 L4. Oligonucleotides ZC1031 (Table 1), ZC1032 (Table 1), ZC1033 (Table 1), and ZC1034 (Table 1) were synthesized on an Applied Biosystems model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. Olgonucleotides ZC1031 and ZC1032 were kinased. Adapters were formed by annealing oligonucleotide ZC1031 with oligonucleotide ZC1034 and by annealing oligonucleotide ZC1032 with oligonucleotide ZC1033 using the method essentially described by Maniatis et al. (ibid.) The vector pUC118 was linearized by digestion with Sal I and Sph I and ligated in a five part ligation with the two

isolated fragments from pAH1 and the annealed pairs of oligonucleotides, ZC1031/ZC1034 and ZC1032/ZC1033. The ligation mixture was transformed into E. coli strain JM83. Plasmid DNA prepared from the resultant transformants were isolated and sequenced to insure a correct fusion. A plasmid having the correct sequence comprising the STE2 gene having the STE2 L4 sequence replaced with a DNA sequence encoding a yeast codon-optimized hamster βAR L4 sequence was designated pHRS4 (Figure 4).

Table 1

ZC87	5'TCC CAG TCA CGA CGT3'
ZC237	5'GCC AGT GAA TTC CAT TGT GTA TTA3'
ZC410	5'CGT AAT ACA GAA TTC CCG GG3'
ZC1031	5' CGC CTT TTG GTG AGT AGC AAC GAT CAT ACC CTT AAC AGC G3'
ZC1032	5' CTG TTA CCA CAA GGA AAC TTG TTG TGA CTT CTT CAC TAA TGC A 3'
ZC1033	5' TTA GTG AAG AAG TCA CAA CAA GTT TCC TTG TGG TAA CAG TCG AT 3'
ZC1034	5' CTG TTA AGG GTA TGA TCG TTG CTA CTC ACC AAA AGG CGA TCG A 3'
ZC1039	5' ACT CTA TTT TAA ATA TCT CTT AAG TAA TTA CTC TTC AG 3'
ZC1040	5'TTA AGT GTT ATG AAG ATG TGG AAC TTC GGT AAC TTC TGG TGT GAA TTC TGG ACT TCT ATC GAC GG 3'
ZC1041	5' CGC CGT CGA TAG AAG TCC AGA ATT CAC ACC AGA AGT TAC CGA AGT TCC ACA TCT TCA TAA CAC 3'
ZC1042	5' ATG TTT ATG GCG CCA CAA ATA TAA T 3'
ZC1413	5' AAT TCT ACA C 3'
ZC1414	5' CAT GGT GTA G 3'

ZC2719 5′ AAT TCA AAA AAT GTC TGA TGC GGC TCC TTC ATT GAG CAA TCT ATT TTA TGA TCC AAC GTA TAA TCC TGG TCA AAG CAC CAT TAA CTA CAC TTC CAT ATA TGG GAA TGG ATC CAC CAT CAC TTT CGA TGA GTT GCA AGG TTT AGT TAA CAG TAC TGT TGG CAT GGG CAT CGT CAT GTC TCT CAT CGT CCT GG 3′

ZC2720 5′ CCA GGA CGA TGA GAG ACA TGA CGA TGC CCA TGC CAA CAG TAC TGT TAA CTA AAC CTT GCA ACT CAT CGA AAG TGA TGG TGG ATC CAT TCC CAT ATA TGG AAG TGT AGT TAA TGG TGC TTT GAC CAG GAT TAT ACG TTG GAT CAT AAA ATA GAT TGC TCA ATG AAG GAG CCG CAT CAG ACA TTT TTT G 3′

ZC2750 5′ AAC ATT GTG CAT GTG ATC CAG GAT AAC CTC ATC CGT AAG GAA GTT TAC ATC CTC CTA AAT TGG ATA GGC TAT GTC AAT TCT GGT TTC AAT CCC CTT ATC TAC TGC CGG GCT GCT AAT AAT GCA 3′

ZC2751 5′ TTA TTA GCA GCC CGG CAG TAG ATA AGG GGA TTG AAA CCA GAA TTG ACA TAG CCT ATC CAA TTT AGG AGG ATG TAA ACT TCC TTA CGG ATG AGG TTA TCC TGG ATC ACA TGC ACA TTG TT 3′

ZC2907 5′ GCC ATT GCC AAG TTC GAG CGT 3′

ZC2909 5′ ATA TAT TCT AGA GCT TTA CAG CAG TGA GTC A 3′

ZC2913 5′ TCG AGA AGA TTC CTT GGT CTC AAG CAG TTC GAT AGT TTA GGC ATC ATC ATG G 3′

ZC2914 5′ GTA CCC ATG ATG ATG CCT AAA CTA TCG AAC TGC TTG AGA CCA AGG AAT CTT C 3′

ZC3120 5′ CAT CAT GGG TAC CTT CAC CCT CTG C 3′

ZC3132 5′ CCT CCT GAA AGG TCG ACC GGT AGA CGA AGA CCA TGA TC 3′

ZC3326 5′ GAA GGA TCC TGA AAT CTG GGC TC 3′

ZC3327 5′ GAT CCT GTA GT 3′

ZC3328 5′ CTA GAC TAC AG 3′

ZC3550 5′ AAT TCA ACG TTG GAT CCA AGA ATC AAA AAT GTC TGA TGC GGC TCC TTC ATT GAT GCA ATC TAT TTT ATG ACG T 3′

ZC3551 5′ CAT AAA ATA GAT TGC TCA ATG AAG GAG CCG CAT CAG ACA TTT TTG ATT CTT GGA TCC AAC GTT G 3′

The sequence encoding the STE2-β_2AR hybrid in plasmid pHRS4 was subcloned into the yeast shuttle vector YEp13 for expression in yeast. Plasmid pHRS4 was digested with Bam HI and Sph I to isolate the 2.3 kb fragment containing the STE2-βAR fusion. Plasmid YEp13 was digested with Bam HI and Sph I to linearize the vector. The linearized vector was ligated with the STE2-βAR fusion fragment. The resultant plasmid was designated pHRS6 (Figure 4).

As shown in Figure 5, the DNA sequence encoding the STE2 L2 was replaced with a DNA sequence encoding a yeast codon-optimized hamster βAR L2 after first inserting unique restriction sites on the borders of the STE2 L2 region. Oligonucleotides ZC1039 (Table 1) and ZC1042 (Table 1) were designed to place an Afl II site at the 5′ border of L2 and a Nar I site at the 3′ border of L2, respectively. Plasmid STE2-SubP #6 was subjected to in vitro mutagenesis using the method essentially described by Kunkel (Proc. Natl. Acad. Sci. USA 82:488-492, 1985). Oligonucleotides ZC1039 and ZC1042 were used as both first and second primers. After mutagenesis, mutants were selected and sequenced to identify plasmids containing both mutant sites. A correct plasmid containing an Afl II site and a Nar I site bordering the STE2 L2 was designated as STE2 #4 1039+1042. The mutagenized STE2 coding sequence present in STE2 #4 1039+1042 was subcloned as a Eco RI-Bgl II fragment into Bam HI-Eco RI linearized pUC9 to generate plasmid pHRS7 (Figure 5).

As shown in Figure 5, the STE2 L2 was replaced by an oligonucleotide adapter containing the sequence for the hamster β_2AR L2 flanked by an Afl II site on the 5′ end and a Nar I site on the 3′ end. Oligonucleotides ZC1041 (Table 1) and ZC1040 (Table 1), which when annealed, encoded a yeast codon-optimized hamster β_2AR L2 adapter, corresponding to nucleotides 280 to 336 of Figure 3, were synthesized on an Applied Biosystems model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. Oligonucleotides ZC1040 and ZC1041 were kinased and annealed using the method essentially described by Maniatis et al. (ibid.). Plasmid pHRS7 was digested with Eco RI and Nar I and with Eco RI and Afl II to isolate the approximately 0.85 kb STE2 fragment and the approximately 4.8 kb STE2+pUC9 fragment, respectively. The ZC1040/ZC1041 kinased adapter, the 0.85 kb Eco RI-Nar I STE2 fragment and the 4.8 kb STE2+pUC9 fragment were joined in a three-part ligation to generate pHRS8, which comprised a DNA sequence encoding STE2 having the STE2 L2 replaced with a yeast codon-optimized hamster β_2AR L2 (Figure 5).

The mutant STE2 gene present in pHRS8 was subcloned into pJH50, a derivative of the yeast vector YEp13. To construct pJH50, YEp13 was modified to destroy the Sal I site near the LEU2 gene by partially digesting YEp13 with Sal I, followed by a complete digestion with Xho I. The 2.0 kb Xho I-Sal I fragment comprising the LEU2 gene and the 8.0 kb linear YEp13 vector fragment were isolated and ligated together. The ligation mixture was transformed into E. coli strain RR1. DNA was prepared from the transformants and was analyzed by digestion with Sal I and Xho I. A clone was isolated which showed a single Sal I site and an inactive Xho I site indicating that the LEU2 fragment had inserted in the opposite orientation relative to the parent plasmid YEp13. The plasmid was designated pJH50.

As shown in Figure 5, plasmid pHRS8 was partially digested with Sal I and completely digested with Sma I to isolate the 2 kb mutant STE2 fragment. This fragment was ligated to pJH50 that had been linearized by digestion with Sal I and Pvu II. The resultant plasmid was designated pHRS5.

A yeast expression vector comprising a DNA construct encoding a STE2-hamster β_2AR fusion with the STE2 L2 and L4 replaced with the hamster β_2AR L2 and L4 was constructed as follows. Plasmid pHRS8 was digested with Sal I and Eco RV to isolate the 1.4 kb STE2-hamster β_2AR L2 fragment. Plasmid pHRS4 was digested with Eco RV and Hind III to isolate the 1 kb fragment comprising the STE2-hamster β_2AR L4 fragment. Plasmid pJH50 was linearized by digestion with Sal I and Hind III and was joined with the 1.4 kb Sal I-Eco RV fragment and the 1 kb Eco RV-Hind III fragment in a three-part ligation. The resulting plasmid was designated pHRS9 (Figure 6).

B. Expression of STE2-Hamster β_2AR Fusions in Yeast

Plasmids pHRS5, comprising the DNA sequence encoding the STE2-hamster β_2AR L2 fusion; pHRS6, comprising the DNA sequence encoding the STE2-hamster β_2AR L4 fusion; and pHRS9, comprising the DNA sequence encoding the STE2-hamster β_2AR L2 + L4 fusion, were transformed into strains XH6-10B (MATa ste2-2 adeX leu2-2,112 lys1 can1) and XH9-5C4 (MATa ste2-1 ade2-1 his3 leu2-2,112 can1) using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on synthetic complete media lacking leucine.

Example 3 - Cloning of a Human β_2-Adrenergic Receptor cDNA

The human β_2AR cDNA was obtained from Brian K. Kobilka (Duke University Medical Center, Durham, NC; Proc. Natl. Acad. Sci. USA 84:46-50, 1987) as a 2.3 kb Eco RI fragment in the vector pSP65 (Figure 8). Briefly, the human βAR cDNA was isolated from a human placental cDNA

library cloned into the phage λgt11. The library was screened using a ^{32}P-labeled 1.3 kb Hind III fragment from the hamster β_2AR genomic clone. Five million recombinants were screened, resulting in the identification of five unique clones with inserts of 1.25 to 2 kb. Restriction enzyme analysis and cross hybridization demonstrated that the smaller clones represented fragments of the larger 2 kb clone. The 2 kb clone was sequenced using the dideoxy chain termination method. The DNA sequence and deduced amino acid sequence for human β_2AR are shown in Figure 7.

Example 4 - Expression of a Human β_2-Adrenergic Receptor in Yeast Cells

The DNA sequence encoding a human β_2AR cDNA obtained from Kobilka (ibid.) was subcloned into a yeast expression vector for expression in yeast as follows.

The TPI1 promoter were obtained from plasmid pTPIC10 (Alber and Kawasaki, J. Mol. Appl. Genet. 1:410-434, 1982) and plasmid pFATPOT (Kawasaki and Bell, EP 171,142; ATCC 20699). Plasmid pTPIC10 was cut at the unique Kpn I site, the TPI1 coding region was removed with Bal-31 exonuclease, and an Eco RI linker (sequence: GGA ATT CC) was added to the 3′ end of the promoter. Digestion with Bgl II and Eco RI yielded a TPI1 promoter fragment having Bgl II and Eco RI sticky ends. This fragment was then joined to plasmid YRp7′ (Stinchcomb et al., Nature 282:39-43, 1979) that had been cut with Bgl II and Eco RI (partial). The resulting plasmid, TE32, was cleaved with Eco RI (partial) and Bam HI to remove a portion of the tetracycline resistance gene. The linearized plasmid was then recircularized by the addition of an Eco RI-Bam HI linker to produce plasmid TEA32. Plasmid TEA32 was digested with Bgl II and Eco RI, and the 900 bp partial TPI1 promoter fragment was gel-purified. Plasmid pIC19H (Marsh et al., Gene 32:481-486, 1984) was cut with Bgl II and Eco RI and the vector fragment was gel purified. The TPI1 promoter fragment was then ligated to

the linearized pIC19H and the mixture was used to transform E. coli RR1. Plasmid DNA was prepared and screened for the presence of a ~900 bp Bgl II-Eco RI fragment. A correct plasmid was selected and designated pICTPIP.

Plasmid pMVR1 was then assembled. Plasmid pIC7 (Marsh et al., ibid.) was digested with Eco RI, the fragment ends were blunted with DNA polymerase I (Klenow fragment), and the linear DNA was recircularized using T4 DNA ligase. The resulting plasmid was used to transform E. coli RR1. Plasmid DNA was prepared from the transformants and was screened for the loss of the Eco RI site. A plasmid having the correct restriction pattern was designated pIC7RI*. Plasmid pIC7RI* was digested with Hind III and Nar I, and the 2500 bp fragment was gel-purified. The partial TPI1 promoter fragment (ca. 900 bp) was removed from pICTPIP using Nar I and Sph I and was gel-purified. The remainder of the TPI1 promoter was obtained from plasmid pFATPOT by digesting the plasmid with Sph I and Hind III, and a 1750 bp fragment, which included a portion of the TPI1 promoter, was gel purifired. The pIC7RI* fragment, the partial TPI1 promoter fragment from pICTPIP, and the fragment from pFATPOT were then combined in a triple ligation to produce pMVR1 (Figure 8).

As shown in Figure 8, a plasmid comprising the β_2AR cDNA sequence in pSP65 was digested with Nco I and Sal I to isolate the 1.7 kb β_2AR fragment. Plasmid pMVR1 was digested with Eco RI and Sal I to isolate the approximately 3.7 kb fragment comprising the TPI1 promoter, the TPI1 terminator and pIC7RI* vector sequences. Synthetic oligonucleotides ZC1413 (Table 1) and ZC1414 (Table 1) were kinased and annealed (using methods essentially described by Maniatis et al. (ibid.)) to form an adapter having a 5′ Eco RI adhesive end and a 3′ Nco I adhesive end. The β_2AR fragment, the pMVR1 fragment and the synthetic adapters were joined by

ligation. A plasmid comprising the TPI1 promoter, β_2AR cDNA, TPI1 terminator and pIC7RI* vector sequences was designated pHRS10 (Figure 8).

The β_2AR expression unit of pHRS10 was subcloned into pJH50 for subsequent transformation into yeast. Plasmid pHRS10 was digested with Xho I and Hind III to isolate the approximately 2.6 kb expression unit comprising the TPI1 promoter, the β_2AR cDNA and the TPI1 terminator. Plasmid pJH50 was digested with Sal I and Hind III to isolate the 11 kb vector fragment. The 2.6 kb pHRS10 fragment and the 11 kb pJH50 fragment were joined in a two part ligation to generate plasmid pHRS11 (Figure 8).

Plasmid pHRS11 was transformed into the Saccharomyces cerevisiae strains XP635-10lac-C1 (MATa leu2-3,112 Δste2 Δbar1::BAR1prom-lacZ gal1), ZY100 (MATa leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1prom-CAT) and ZY400 (MATa leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1prom-CAT Δmnn9::URA3) using the method generally described by Beggs (ibid.). Transformants were selected for their ability to grow in synthetic complete media lacking the amino acid leucine.

Transformants were assayed for the presence of biologically active β_2AR by radio-ligand binding using an assay adapted from the method described by Dixon et al. (ibid., 1987). The assay relies upon the displacement of labeled iodocyanopindolol (^{125}I-CYP), which binds nonspecifically to cell membranes in addition to β_2ARs and is considered a β_2AR antagonist, from the yeast-expressed β_2AR receptors by a β_2AR ligand. Plasmid pHRS11 transformants were inoculated into 250 ml of -LEUD medium (Table 2) and grown overnight at 30°C. The overnight cultures were diluted 1:2 into fresh -LEUD medium and were grown for two hours at 30°C. The log phase cells were pelleted by centrifugation, and the cells were washed in 20 ml of Binding Buffer (Table 2). The A_{660} was taken of a 1:100 dilution to estimate the density of the cells.

Table 2

Media Recipes

-LeuThrTrp Amino Acid Mixture

4 g adenine
3 g L-arginine
5 g L-aspartic acid
2 g L-histidine free base
6 g L-isoleucine
4 g L-lysine-mono hydrochloride
2 g L-methionine
6 g L-phenylalanine
5 g L-serine
5 g L-tyrosine
4 g uracil
6 g L-valine

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

-LEUD

20 g glucose
6.7 g Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, MI)
0.6 g -LeuThrTrp Amino Acid Mixture

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan.

Binding Buffer

15 mM Tris, pH 7.5
12.5 mM $MgCl_2$
0.3 M EDTA

To measure receptor-bound ligand, the displacement of receptor-bound ^{125}I-CYP was measured by subtracting the ^{125}I-CYP counts bound in the presence of a known β_2AR ligand, such as alprenolol (ALP), from the counts of nonspecifically bound ^{125}I-CYP. Competition binding experiments using β_2AR agonists and antagonists were measured by subtracting the ^{125}I-CYP counts bound in the presence of serially diluted agonist or antagonist from the ^{125}I-CYP counts bound in the presence of a saturating concentration of ALP.

Saturation binding experiments were carried out as follows. Increasing concentrations of ^{125}I-CYP (New England Nuclear) were incubated with Binding Buffer containing 3 x 10^8 cells in the presence or absence of 10 μM ALP (Sigma, St. Louis, MO). The mixtures were incubated at 22°C for one half hour. During the incubation, the mixture was vortexed one time. One ml aliquots of the mixture were loaded onto glass fiber G/FC Whatman filters. Cells were washed with ten volumes of Binding Buffer by suction. Filters were then counted on a gamma counter. Bound counts indicated the amount of bound ^{125}I-CYP. Receptor-bound counts, determined by the equation below, were plotted as a function of the log of the concentration. The concentration of ALP found to saturate the β_2ARs expressed by the pHRS11 transformants was found to be at least 10 μM. One hundred times the saturation concentration of ALP was subsequently used for competition binding experiments.

$$[^{125}\text{I-CYP}] - [\text{ALP} + {}^{125}\text{I-CYP}] = \text{receptor-bound counts}$$

where

$[^{125}\text{I-CYP}]$ = total bound counts and

$[\text{ALP} + {}^{125}\text{I-CYP}]$ = nonspecifically bound counts

Competition binding assays with isoproterenol, epinephrine and norepinephrine were carried out on the

transformants as described above, except that a control tube comprising a saturating concentration of alprenolol of 1 mM + 75 pM CYP added to 3x 10^8 cells in 3 ml of Binding Buffer was prepared to determine the total availability of G protein-coupled receptor present on the host cells. In addition, assay tubes containing serial dilutions of isoproterenol, epinephrine, and norepinephrine (Sigma Chemical Co., St Louis, MO) mixed with 75 pM ^{125}I-CYP were prepared. The percent maximal for the ligands isoproterenol, epinephrine, and norepinephrine were plotted as a function of the negative log of the concentration of the ligand. The percent of maximal for each ligand was determined using the equation below.

$$([^{125}\text{I-CYP} + \text{ligand}] - [\text{exALP} + {}^{125}\text{I-CYP}]) + ([^{125}\text{I-CYP}] - [\text{exALP} + {}^{125}\text{I-CYP}]) \times 100 = \% \text{ maximal}$$

where

$[^{125}\text{I-CYP}]$ = total bound counts

exALP = an excess concentration of ALP capable of competing with ^{125}I-CYP for all available receptor

$[\text{exALP} + {}^{125}\text{I-CYP}]$ = nonspecifically bound counts in the presence of excess ALP

$[^{125}\text{I-CYP} + \text{ligand}]$ = nonspecifically bound counts in the presence of a concentration of ligand

Representative competition binding curves for ligand binding assays using isoproterenol, epinephrine and norepinephrine and ZY100 cells transformed with pHRS11 are shown in Figures 10 and 11.

Example 5 - Construction and Expression of Human β2-Adrenergic-STE2 Hybrid Receptors

A. Construction of pHRS17

A DNA construct comprising a DNA sequence encoding a human β-adrenergic-STE2 receptor hybrid receptor was constructed by replacing the DNA sequence encoding the extracellular amino-terminal domain of the human β_2AR with a DNA sequence encoding the extracellular amino-terminal domain of the STE2 gene product. To construct plasmid pHRS16, oligonucleotides ZC2719 and ZC2720 were designed to encode a 5′ end by an Eco RI adhesive end followed by the extracellular amino-terminal domain of the STE2 gene product containing nucleotides 1 to 147 of Figure 9 joined to nucleotides 103 to 136 of Figure 7. Oligonucleotides were synthesized and phosphorylated on an Applied Biosystems model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. The kinased oligonucletides are annealed

using the method essentially described by Maniatis et al. (ibid.).

The plasmid comprising the β_2AR cDNA sequence in pSP65 is digested with Bal I and Sal I to isolate the 1.8 kb fragment comprising the β_2AR coding sequence from nucleotide 137 to 1242 of Figure 7. Plasmid pMVR1 is digested with Eco RI and Sal I to isolate the 3.7 kb fragment comprising the TPI1 promoter, TPI1 terminator and pIC7RI* vector sequences. The ZC2719/ZC2720 oligonucleotide adapter, the β_2AR fragment and the pMVR1 vector fragment are joined in a four-part ligation. The resultant plasmid was designated pHRS16.

The expression unit from pHRS16, comprising the TPI1 promoter, the STE2-β_2AR coding sequence and the TPI1 terminator, are subcloned into the yeast shuttle vector pJH50. Plasmid pHRS16 is diegested with Hind III and Xho I to isolate the 2.8 kb expression unit. Plasmid pJH50 is digested with Sal I and Hind III to isolate the vector fragment. The pHRS16 and pJH50 fragments are joined by ligation, and the resulting plasmid is designated pHRS17.

B. Construction of pHRS18

A DNA construct comprising a DNA sequence encoding a hybrid human β_2AR-STE2 receptor is constructed from a human β_2AR coding sequence by replacing the DNA sequence encoding human β_2AR carboxy-terminal internal effector domain with the DNA sequence encoding the corresponding domain of the Saccharomyces cerevisiae STE2 gene product. Plasmid pHRS18 is constructed as follows.

Synthetic oligonucleotides were designed to encode a β_2AR-STE2 adapter comprising the nucleotide sequence of Figure 6 from 877 to 985 joined the nucleotide sequence of Figure 8 from 892 to 903 flanked by a 3′ Nsi I adhesive end. The oligonucleotides were synthesized and phosphorylated on an Applied Biosystems model 380A DNA synthesizer and purified by acrylamide gel electrophoresis. The oligonucleotides are annealed using

the method essentially described by Maniatis et al. (ibid.).

Plasmid pHRS10 is digested with Xho I and Hpa I to isolate the 1.7 kb fragment comprising the TPI1 promoter and 5′ β_2AR cDNA sequences. Plasmid pAH3 is digested with Nsi I and Hind III to isolate the kb fragment comprising the sequence encoding the 3′ portion of the carboxy-terminal internal effector domain and the associated STE2 3′ untranslated sequences. Plasmid pJH50 is digested with Sal I and Hind III to isolate the vector fragment. The ZC2750/ZC2751 adapter, the pHRS10 fragment, the STE2 fragment from pAH3 and the pJH50 vector fragment are joined in a four-part ligation. The resultant plasmid is designated pHRS18.

C. Construction of pHRS40

A DNA construct comprising a DNA sequence encoding a human β_2AR-STE2 receptor hybrid having a portion of the STE2 extracellular amino terminal and third internal domains (EATD and 3-ID, respectively) was constructed by replacing the DNA sequences encoding the EATD and 3-ID of the human β_2AR with DNA sequences encoding the EATD and 3-ID of the STE2 gene product.

Plasmid pHRS20 was constructed using oligonucleotide pairs ZC3120 and ZC2909 and ZC3132 and ZC2907 (Table 1) in polymerase chain reactions to generate β_2AR fragments having unique restriction sites flanking the third internal domain. The third internal domain of STE2 was generated from an oligonucleotide adapter formed by annealing oligonucleotides ZC2913 with ZC2914 (Table 1). The β_2AR coding sequence used as a template in the polymerase chain reactions was obtained from Hind III-digested pHRS10 (Example 4, Figure 8). Hind III digestion of pHRS10 generates two fragments one comprises the TPI1 promoter, β_2AR coding sequence, TPI1 terminator and one comprises pIC7RI* vector sequences.

A fragment encoding the 3′ coding sequence of the β_2AR from nucleotides 831 to 1242 of Figure 7 and

having an Asp 718 site within TMD6 and an Xba I site 3′ to the β_2AR stop codon was generated by PCR amplification using Hind III-digested pHRS10 as a template. One nanogram of Hind III-digested pHRS10 was amplified using the GeneAmp kit (Perkin Elmer Cetus PCR, Norwalk, CT) using 100 pmoles each of oligonucleotides ZC3120 and ZC2909 in a 100 µl reaction volume under conditions described by the manufacturer. After 30 cycles (30 seconds at 94°C, 30 seconds at 45°C and two minutes at 72°C) followed by an incubation for seven minutes at 72°C, the samples were cooled to 4°C and electrophoresed in an agarose gel. The PCR-generated fragments were gel purified and were digested with Asp 718 and Xba I to isolate the 0.42 kb fragment comprising the β_2AR coding sequence from the β_2AR TMD6 through the stop codon.

A fragment encoding a portion of the 5′ coding sequence of the β_2AR from nucleotides 169 to 676 of Figure 7 and having a Pst I site at nucleotide 194 and a Sal I site within TMD5 was generated by PCR amplification from Hind III-digested pHRS10 as described above using 100 pmoles each of oligonucleotides ZC3132 and ZC2907 (Table 1) in a 100 µl reaction volume using the conditions described above. The gel-purified fragment was digested with Pst I and Sal I to isolate the 0.464 kb fragment comprising the β_2AR coding sequence from the initiation codon to Sal I site within TMD5.

Oligonucleotides ZC2913 and ZC2914, synthesized as described previously, were designed to form when annealed a 54 bp Xho I-Asp 718 adapter encoding the STE2 3-ID. Oligonucleotides ZC2913 and ZC2914 were kinased and annealed essentially as described by Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, New York, 1989, which is incorporated by reference herein).

Plasmid pHRS20 was assembled by ligating the 0.464 kb Pst I-Sal I fragment comprising a portion of the 5′ β_2AR coding sequence, the ZC2913/ZC2914 adapter

encoding the STE2 3-ID, and the 0.42 kb Asp 718-Xba I fragment comprising the 3′ β_2AR coding sequence with the 3.9 kb Pst I-Xba I fragment of pHRS10 comprising the TPI1 promoter, the 5′ β_2AR coding sequence, pIC7RI* vector sequences, and the TPI1 terminator. Plasmid pHRS20 was confirmed by restriction analysis. Sequence analysis of pHRS20 disclosed a A -> G silent mutation at corresponding to nucleotide 918 of Figure 7.

The sequence encoding the β_2AR EATD was replaced with a portion of the STE2 EATD by first digesting pHRS20 with Aat II and Xho I to isolate the 1.15 kb fragment comprising the hybrid β_2AR-STE2 receptor coding sequence and the TPI1 terminator. Oligonucleotides ZC3550 and ZC3551 (Table 1), synthesized as described previously, were kinased and annealed to form an Eco RI-Aat II adapter encoding the first fourteen amino acids of STE2 (nucleotides 1-42 of Figure 9). The 1.15 kb pHRS20 fragment and the ZC3550/ZC3551 adapter were ligated with Sal I-Eco RI linearized pUC18, and the resultant plasmid, which was confirmed by restriction and sequence analysis, was designated pHRS45.

The β_2AR-STE2 coding sequence present in pHRS45 is sublconed into the yeast expression vector pJH50. Plasmid pHRS45 is digested with Eco RI and Hind III to isolate the 1.21 kb fragment comprising the β_2AR-STE2 coding sequence and TPI1 terminator.

The ADH2 promoter is obtained from plasmid p410WT as a Bam HI-Eco RI fragment. The ADH2 promoter present in p410WT derived from pBR322-ADR2-BSa (Williamson et al., ibid.). The 2.2 kb Bam HI fragment containing the wild-type ADH2 structural gene and 5′ flanking sequences from pBR322-ADR2-BSa was ligated with M13mp19 which had been linearized with Bam HI. The orientation of the insert was determined by restriction analysis. Using site-specific in vitro mutagenesis (Zoller et al., DNA 3: 479-488, 1984) and ZC237 (Table 1) as the second primer, the structural portion of the ADH2 gene was removed from

the ADH2 insert in M13mp19 and joined to the 5′ flanking sequence, including the translation start signal, with the Eco RI site of the m13mp19 polylinker. Replicative form DNA prepared from a positive phage clone was digested with Bam HI and Eco RI to isolate the 1.2 kb promoter fragment. This 1.2 kb fragment was ligated into pUC13 which had been linearized with Bam HI and Eco RI to generate plasmid p237-Wt.

The ADH2 promoter was then fused to the codon for the first amino acid of the mature form of α-1-antitrypsin (AAT) in the plasmid pAT-1. Plasmid pAT-1 comprises the expression unit of the ADH2 promoter from p237-Wt joined to the AAT cDNA-TPI1 terminator sequence from the plasmid pMVR1 (Example 4). These sequences were inserted into a portion of the vector pCPOT. (Plasmid pCPOT has been deposited with ATCC as an E. coli strain HB101 transformant and has been assigned accession number 39685. It comprises the entire 2 micron plasmid DNA, the leu2-d gene, pBR322 sequences and the Schizosaccharomyces pombe POT1 gene.) Plasmid pCPOT was digested with Bam HI and Sal I to isolate the approximately 10 kb linear vector fragment. Plasmid pMVR1 was digested with Eco RI and Xho I to isolate the 1.5 kb α-1-antitrypsin cDNA-TPI1 terminator fragment. The 1.2 kb ADH2 promoter fragment was isolated from p237-Wt as a Bam HI-Eco RI fragment and was joined with the 1.5 kb a-1-antitrypsin cDNA-TPI1 terminator fragment and the linearized pCPOT in a three-part ligation to yield a plasmid designated pAT-1.

The ADH2 promoter from plasmid pAT-1 was modified to create a "universal" promoter by removing the ADH2 translation start site and the pUC18 polylinker sequences found in pAT-1 (Figure 4). Plasmid pAT-1 was digested with Sph I and Bam HI to isolate the 190 bp partial ADH2 promoter fragment. This fragment was ligated into M13mp18 linearized with Bam HI and Sph I. The resulting construction was subjected to in vitro mutagenesis (Zoller et al., ibid.) using ZC410 (Table 1)

as the mutagenic primer and ZC87 as the second primer. The mutagenesis using ZC410 replaces the ADH2 translation start signal and pUC18 polylinker sequences with a single Eco RI site fused to the m13mp18 polylinker at the Sma I site. Positive clones were confirmed by dideoxy sequencing through the fusion point. For ease of manipulation, the mutagenized partial ADH2 promoter fragment was subcloned as a 175 bp Sph I-Eco RI fragment into pUC19 which had been linearized by Sph I and Eco RI. The resulting plasmid, designated p410ES, contained the 3′ most 175 bp of the ADH2 promoter. The wild-type ADH2 promoter was regenerated using the partial ADH2 promoter fragment from p410ES. Plasmid p410ES was digested with Sph I and Eco RI to isolate the 175 bp partial ADH2 promoter fragment. This fragment was joined with a 1 kb Bam HI-Sph I fragment derived from pBR322-ADR2-BSa in a three-part ligation into pUC13 which had been linearized by digestion with Bam HI and Eco RI. The 1 kb fragment derived from pBR322-ADR2-BSa contained sequences that are homologous with wild-type ADH2 promoter sequence. The plasmid that resulted from the three-part ligation was confirmed by restriction analysis and designated p410WT.

Plasmid p410WT is digested with Bam HI and Eco RI to isolate the 1.2 kb ADH2 promoter fragment. The 1.2 kb Bam HI-Eco RI ADH2 fragment and the 1.21 kb Eco RI-Hind III fragment from pHRS45 are ligated with Hind III-Bam HI linearized pJH50. The resultant plasmid is designated pHRS40. Plasmid pHRS40 is transformed into S. cerevisiae strains ZY100 and XP636-10lac-C1 and transformants are assayed for the presence of biologically active β_2AR as described above.

D. The Construction of pHRS41

The β_2AR EATD was replaced with a portion of the STE2 EATD using a DNA construct wherein the 3′ non-coding region of the β_2AR was removed. The truncated β_2AR was generated by PCR amplification of a fragment using

oligonucleotides ZC2909 and ZC2907 (Table 1) and Hind III-digested pHRS10 as a template. Using the GeneAmp Kit (Perkin Elmer Cetus), one nanogram of Hind III-digested pHRS10 and 20 pmoles each of oligonucleotides ZC2909 and ZC2907 were used to amplify a fragment using the conditions set forth above. After amplification, using the conditions described above, the fragment was purified by agarose gel electrophoresis. The gel-purified fragment was digested with Pst I and Xba I to isolate the 1.06 kb Pst I-Xba I fragment comprising the 3′ portion of the β_2AR having an Xba I site just 3′ to the stop codon. The 1.06 kb fragment was ligated with Pst I-Xba I digested pHRS10 comprising the 5′ β_2AR coding sequence, the TPI1 promoter, pIC7RI* vector sequence and the TPI1 terminator. The resulting plasmid was designated pHRS22.

The β_2AR EATD present in plasmid pHRS22 is replaced with a portion of the STE2 EATD and the expression unit is subcloned into a yeast expression vector. Plasmid pHRS22 is digested with Pst I and Hind III to isolate the 1.1 kb fragment comprising the 3′ β_2AR coding region. Plasmid pHRS40 is digested with Sal I and Pst I to isolate the ADH2 promoter and 5′ STE2 EATD-β_2AR coding region. The 1.7 kb Sal I-Pst I fragment and the 1.1 kb Pst I-Hind III fragment are ligated with Sal I-Hind III digested pJH50 to generate pHRS41. Plasmid pHRS41 is transformed into S. cerevisiae strains ZY100 and XP636-10lac-C1 and transformants are assayed for the presence of biologically active β_2AR as described above.

E. Construction of pHRS42 and pHRS43

The C-terminal internal domains (C-IDs) of the β_2AR coding sequence present in pHRS22 and the β_2AR-STE2 coding sequence present in pHRS20 were removed by the PCR amplification of fragments from pHRS22 and pHRS20 which inserted a Bam HI site between nucleotides 999 and 1006 of Figure 7 and truncated the β_2AR sequence after TMD7. An in-frame stop codon was inserted using an oligonucleotide

adapter prepared by annealing kinased oligonucleotides ZC3327 and ZC3328 (Table 1).

Two polymerase chain reactions were set up using the GeneAmp Kit (Perkin Elmer Cetus) either 1 µl of a pHRS22 plasmid preparation or 1 µl of a pHRS20 plasmid preparation. One hundred picomoles each of ZC2907 and ZC3326 were added to each reaction. After 30 cycles (30 seconds at 94°C, 30 seconds at 45°C and two minutes at 72°C) followed by one cycle (30 seconds at 94°C, 30 seconds at 45°C and seven minutes at 72°C), the samples were cooled to 4°C and electrophoresed in an agarose gel. The PCR-generated fragments were gel purified and were digested with Pst I and Bam HI to isolate the .8 kb fragment from the pHRS22 amplification and the .6 kb fragment from the pHRS20 amplification.

Oligonucleotides ZC3327 and ZC3328, synthesized as described above, were designed to create, when annealed, a Bam HI-Xba I site adapter encoding an in-frame stop codon for the β_2AR and β_2AR-STE2 PCR-generated fragments. Oligonucleotides ZC3327 and ZC3328 were kinased and annealed as described above.

The 0.8 kb fragment generated from pHRS22 and the 0.6 kb fragment generated from pHRS20 were each ligated with the ZC3327/ZC3328 adapter and the Pst I-Xba I fragment of pHRS10 comprising the 5' β_2AR coding sequence, the TPI1 promoter, pIC7RI* vector sequence and the TPI1 terminator. A plasmid comprising the TPI1 promoter, a truncated β_2AR sequence, the TPI1 terminator and pIC7RI* vector sequences was designated pHRS31. A plasmid comprising the TPI1 promoter, the truncated β_2AR-STE2 sequence, the TPI1 terminator and pIC7RI* vector sequences was designated pHRS32.

The β_2AR EATDs of pHRS31 and pHRS32 are replaced with a portion of the STE2 EATD and the expression units are subcloned into a yeast expression vector. Plasmids pHRS31 and pHRS32 are each digested with Pst I and Hind III to isolate the .9 kb and .77 kb fragments,

respectively, comprising the 3′ β_2AR or β_2AR-STE2 coding sequence and TPI1 terminator. Plasmid pHRS40 is digested with Sal I and Pst I to isolate the 1.7 kb fragment comprising the ADH2 promoter joined to the STE2 EATD-β_2AR coding sequence. The Pst I-Hind III fragments isolated from pHRS31 and pHRS32 are each ligated with the 1.7 kb Sal I-Pst I fragment from pHRS45 and Sal I- Hind III linearized pJH50. A plasmid resulting from the ligation of the pHRS31 fragment with the pHRS45 fragment is designated pHRS42. A plasmid resulting from the ligation of the pHRS32 fragment and the pHRS45 fragment is designated pHRS43. Plasmids pHRS42 and pHRS43 are transformed into S. cerevisiae strains ZY100 and XP635-10lac-C1. The transformants are assayed for the presence of biologically active β_2AR as described above.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

1. A DNA sequence encoding a biologically active hybrid G protein-coupled receptor, said receptor comprising a mammalian G protein-coupled receptor having at least one domain other than the ligand-binding domain replaced with the corresponding domain of a yeast G protein-coupled receptor.

2. The DNA sequence of claim 1 wherein the yeast G protein-coupled receptor is selected from the group consisting of the Saccharomyces cerevisiae STE2 gene product, the Saccharomyces cerevisiae STE3 gene product and the Saccharomyces kluyveri STE2 gene product.

3. The DNA sequence of claim 1 wherein the yeast G-protein-coupled receptor is the Saccharomyces cerevisiae STE2 gene product.

4. The DNA sequence of claim 1 wherein the mammalian G protein-coupled receptor is selected from the group consisting of β-adrenergic receptors, α-adrenergic receptors, muscarinic receptors, angiotensin receptors, substance K receptors and rhodopsin receptors.

5. The DNA sequence of claim 1 wherein the mammalian G protein-coupled receptor is selected from the group consisting of human β_2-adrenergic receptors, human β_1-adrenergic receptors, human α-adrenergic receptors, human muscarinic receptors, human rhodopsin receptors, human angiotensin receptors and human substance K receptors.

6. The DNA sequence of claim 1 wherein the domain of the mammalian G protein-coupled receptor is selected from the group consisting of at least a portion of the extracellular amino-terminal domain, the effector domain, the third internal effector domain and the carboxy-terminal internal effector domain.

7. The DNA sequence of claim 1 wherein an extracellular amino-terminal domain and an effector domain of the mammalian G protein-coupled receptor are replaced with an extracellular amino-terminal domain and an effector domain, respectively, of a yeast G protein-coupled receptor.

8. The DNA sequence of claim 7 wherein the effector domain of the mammalian G protein-coupled receptor selected from the group consisting of a carboxy-terminal internal effector domain, a third internal effector domain, and a carboxy-terminal internal effector domain and a third internal effector domain is replaced with a carboxy-terminal internal effector domain, a third internal effector domain, and a carboxy-terminal internal effector domain and a third internal effector domain, respectively, of a yeast G protein-coupled receptor.

9. A DNA construct capable of directing the expression of a biologically active hybrid G protein-coupled receptor in a yeast cell, comprising the following operatively linked elements:

a transcriptional promoter;

a DNA sequence according to any one of claims 1-8; and

a transcriptional terminator.

10. A yeast host cell transformed with a DNA construct according to claim 9.

11. The yeast host cell of claim 10 wherein the yeast host cell is a Saccharomyces cerevisiae cell.

12. The yeast host cell of claim 11 wherein the yeast host cell contains a genetically defective STE2 or STE3 gene.

13. The yeast host cell of claim 11 wherein the yeast host cell is a mating-type α haploid cell.

14. The yeast host cell of claim 11 wherein the yeast host cell is a mating-type a haploid cell.

15. The yeast host cell of claim 14 wherein the yeast host cell does not contain a functional BAR1 gene.

16. The yeast host cell of claim 11 wherein said host cell is also transformed with a second DNA construct comprising a mating-type specific gene promoter operatively linked to an indicator DNA sequence, and wherein said step of detecting comprises detecting the expression of said indicator DNA sequence.

17. The yeast host cell of claim 14 wherein the yeast host cell is transformed with a second DNA construct comprising the BAR1 promoter operatively linked to the E. coli lacZ coding sequence, and the second DNA construct is integrated at the BAR1 locus.

18. A method for detecting the presence of ligand in a test sample, comprising the steps of:

a) exposing a culture of yeast host cells according to any one of claims 10-17 to the test sample under suitable conditions to allow binding of ligand to the hybrid G protein-coupled receptor; and

b) detecting a biological response of the host cell and therefrom determining the presence of the ligand.

19. The method of claim 18 wherein the cells are suspended in an agar overlay on top of an appropriate solid growth medium.

20. The method of claim 19 wherein the agar overlay includes one or more wells and the step of exposing comprises:
filling the wells with the test sample.

21. The method of claim 19 wherein the step of exposing comprises placing a filter saturated with the test sample onto the agar overlay.

22. The method of claim 19 wherein the agar overlay contains an agonist.

23. The method of claim 18 wherein the yeast host cells are mating-type a haploid cells transformed with DNA construct comprises a hybrid G protein-coupled receptor, wherein said receptor comprises a mammalian G protein-coupled receptor having at least one domain replaced with a corresponding domain of a yeast G protein-coupled receptor selected from the group consisting of the Saccharomyces cerevisiae STE2 gene product and the Saccharomyces kluyveri STE2 gene product, and wherein the step of detecting comprises detecting the presence of a halo of host cells arrested in the G1 phase of cell division.

24. The method of claim 22 wherein the yeast host cells are mating-type a haploid cells transformed with DNA construct comprises a hybrid G protein-coupled receptor, wherein said receptor comprises a mammalian G protein-coupled receptor having at least one domain replaced with a corresponding domain of a yeast G protein-coupled receptor selected from the group consisting of the Saccharomyces cerevisiae STE2 gene product and the Saccharomyces kluyveri STE2 gene product, and wherein the step of detecting comprises detecting the presence of a halo of host cell colonies.

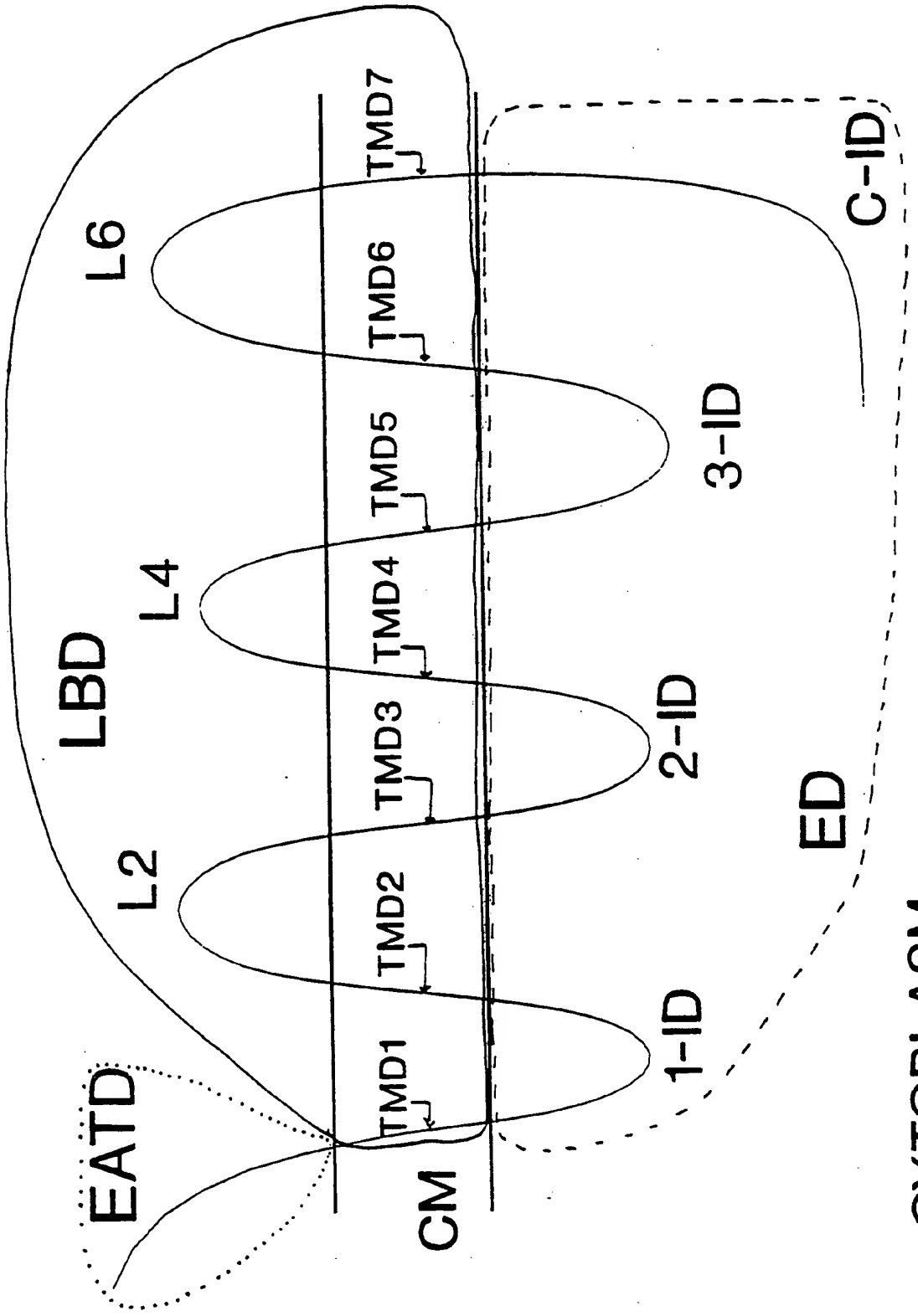
EATD
L2
LBD
L4
L6
TMD1
TMD2
TMD3
TMD4
TMD5
TMD6
TMD7
CM
1-ID
2-ID
3-ID
ED
C-ID
CYTOPLASM

Figure 1.

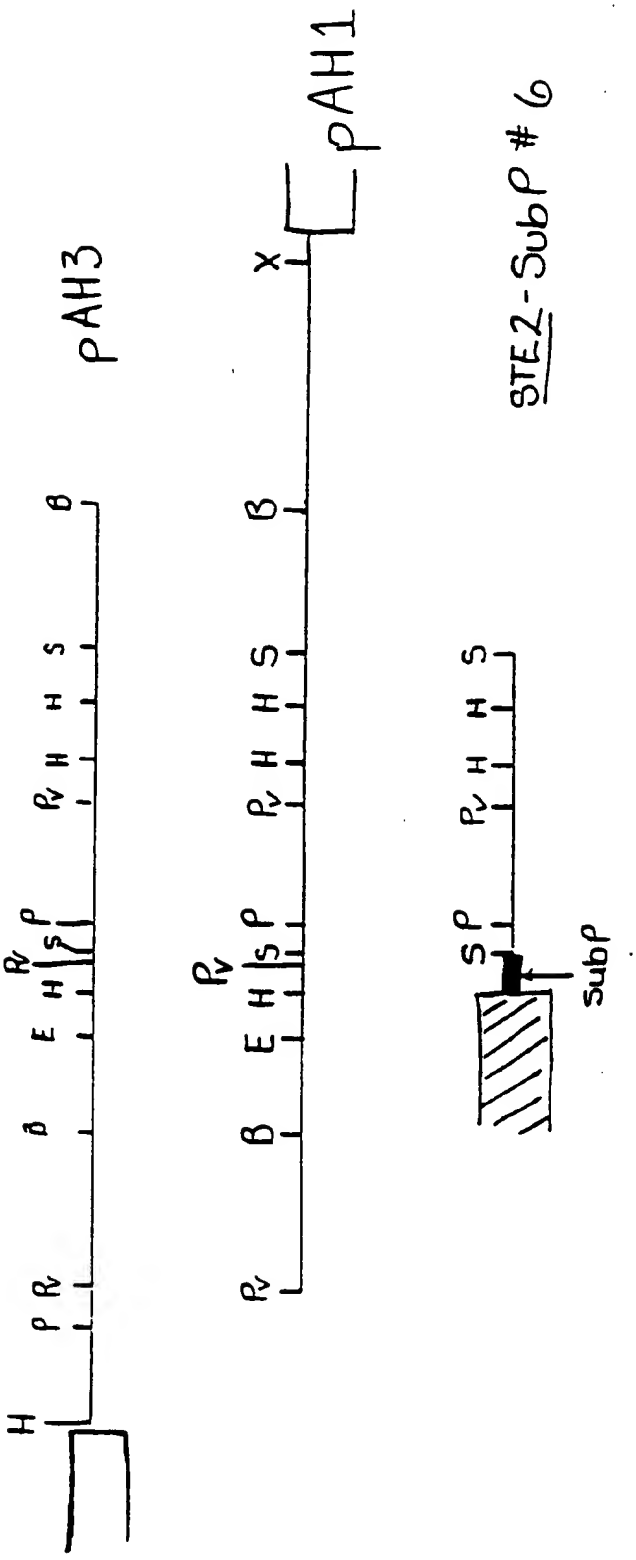

Figure 2

Figure 3

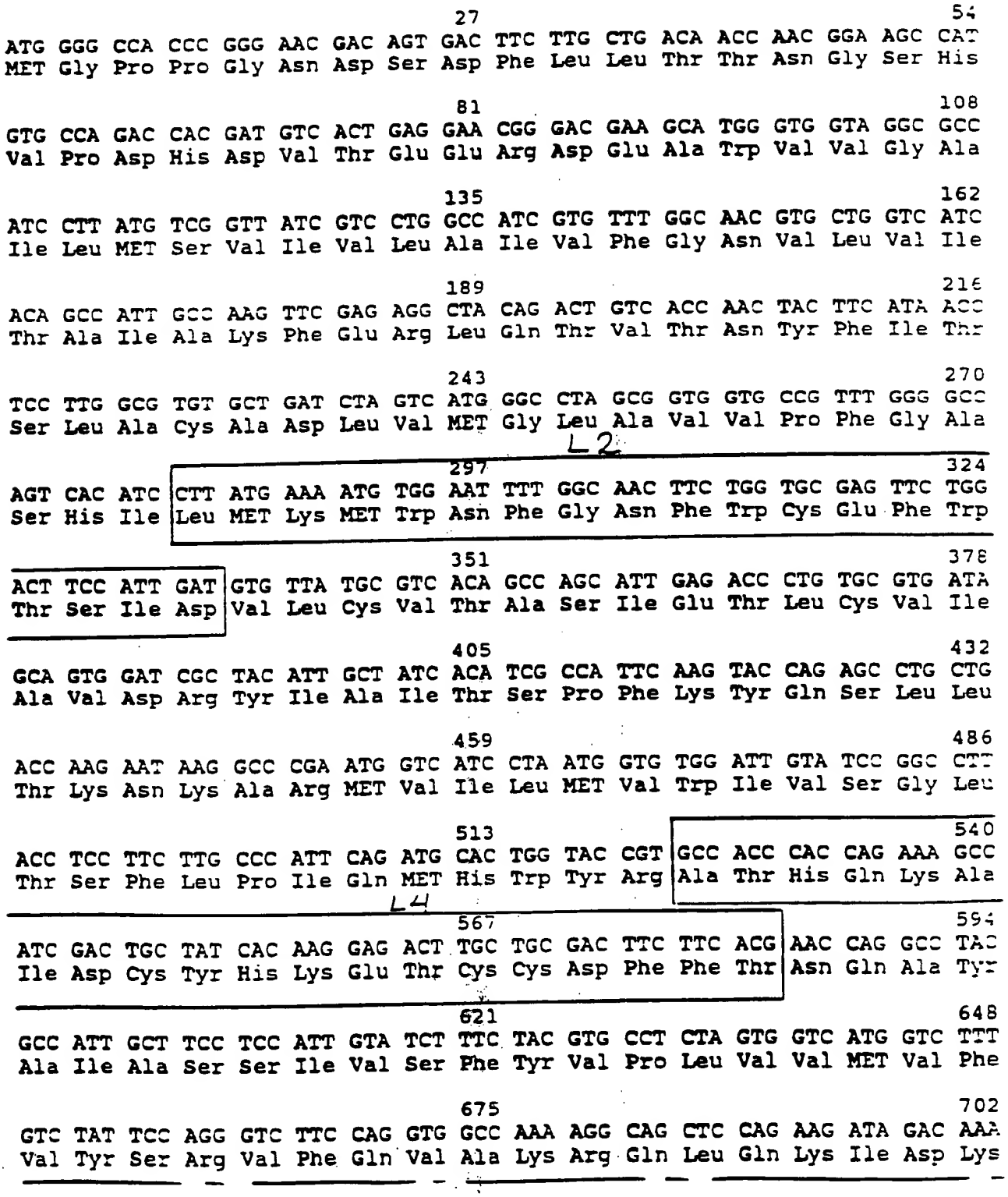
27
54
ATG GGG CCA CCC GGG AAC GAC AGT GAC TTC TTG CTG ACA ACC AAC GGA AGC CAT
MET Gly Pro Pro Gly Asn Asp Ser Asp Phe Leu Leu Thr Thr Asn Gly Ser His
81
108
GTG CCA GAC CAC GAT GTC ACT GAG GAA CGG GAC GAA GCA TGG GTG GTA GGC GCC
Val Pro Asp His Asp Val Thr Glu Glu Arg Asp Glu Ala Trp Val Val Gly Ala
135
162
ATC CTT ATG TCG GTT ATC GTC CTG GCC ATC GTG TTT GGC AAC GTG CTG GTC ATC
Ile Leu MET Ser Val Ile Val Leu Ala Ile Val Phe Gly Asn Val Leu Val Ile
189
216
ACA GCC ATT GCC AAG TTC GAG AGG CTA CAG ACT GTC ACC AAC TAC TTC ATA ACC
Thr Ala Ile Ala Lys Phe Glu Arg Leu Gln Thr Val Thr Asn Tyr Phe Ile Thr
243
270
TCC TTG GCG TGT GCT GAT CTA GTC ATG GGC CTA GCG GTG GTG CCG TTT GGG GCC
Ser Leu Ala Cys Ala Asp Leu Val MET Gly Leu Ala Val Val Pro Phe Gly Ala
L2
297
324
AGT CAC ATC CTT ATG AAA ATG TGG AAT TTT GGC AAC TTC TGG TGC GAG TTC TGG
Ser His Ile Leu MET Lys MET Trp Asn Phe Gly Asn Phe Trp Cys Glu Phe Trp
351
378
ACT TCC ATT GAT GTG TTA TGC GTC ACA GCC AGC ATT GAG ACC CTG TGC GTG ATA
Thr Ser Ile Asp Val Leu Cys Val Thr Ala Ser Ile Glu Thr Leu Cys Val Ile
405
432
GCA GTG GAT CGC TAC ATT GCT ATC ACA TCG CCA TTC AAG TAC CAG AGC CTG CTG
Ala Val Asp Arg Tyr Ile Ala Ile Thr Ser Pro Phe Lys Tyr Gln Ser Leu Leu
459
486
ACC AAG AAT AAG GCC CGA ATG GTC ATC CTA ATG GTG TGG ATT GTA TCC GGC CTT
Thr Lys Asn Lys Ala Arg MET Val Ile Leu MET Val Trp Ile Val Ser Gly Leu
513
540
ACC TCC TTC TTG CCC ATT CAG ATG CAC TGG TAC CGT GCC ACC CAC CAG AAA GCC
Thr Ser Phe Leu Pro Ile Gln MET His Trp Tyr Arg Ala Thr His Gln Lys Ala
L4
567
594
ATC GAC TGC TAT CAC AAG GAG ACT TGC TGC GAC TTC TTC ACG AAC CAG GCC TAC
Ile Asp Cys Tyr His Lys Glu Thr Cys Cys Asp Phe Phe Thr Asn Gln Ala Tyr
621
648
GCC ATT GCT TCC TCC ATT GTA TCT TTC TAC GTG CCT CTA GTG GTC ATG GTC TTT
Ala Ile Ala Ser Ser Ile Val Ser Phe Tyr Val Pro Leu Val Val MET Val Phe
675
702
GTC TAT TCC AGG GTC TTC CAG GTG GCC AAA AGG CAG CTC CAG AAG ATA GAC AAA
Val Tyr Ser Arg Val Phe Gln Val Ala Lys Arg Gln Leu Gln Lys Ile Asp Lys

Figure 3

```
                                    729                                 756
TCT GAG GGA AGA TTC CAC TCC CCA AAC CTC GGC CAG GTG GAG CAG GAT GGG CGG
Ser Glu Gly Arg Phe His Ser Pro Asn Leu Gly Gln Val Glu Gln Asp Gly Arg

                                    783                                 810
AGT GGG CAC GGA CTC CGA AGG TCC TCC AAG TTC TGC TTG AAG GAG CAC AAA GCC
Ser Gly His Gly Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys Ala

                                    837                                 864
CTC AAG ACT TTA GGC ATC ATC ATG GGC ACA TTC ACC CTC TGC TGG CTG CCC TTC
Leu Lys Thr Leu Gly Ile Ile MET Gly Thr Phe Thr Leu Cys Trp Leu Pro Phe

                                    891                                 918
TTC ATT GTC AAC ATC GTG CAC GTG ATC CAG GAC AAC CTC ATC CCT AAG GAA GTT
Phe Ile Val Asn Ile Val His Val Ile Gln Asp Asn Leu Ile Pro Lys Glu Val

                                    945                                 972
TAC ATC CTC CTT AAC TGG TTG GGC TAT GTC AAT TCT GCT TTC AAT CCC CTC ATC
Tyr Ile Leu Leu Asn Trp Leu Gly Tyr Val Asn Ser Ala Phe Asn Pro Leu Ile

                                    999                                1026
TAC TGT CGG AGT CCA GAT TTC AGG ATT GCC TTC CAG GAG CTT CTA TGC CTC CGC
Tyr Cys Arg Ser Pro Asp Phe Arg Ile Ala Phe Gln Glu Leu Leu Cys Leu Arg

                                   1053                                1080
AGG TCT TCT TCA AAA GCC TAT GGG AAC GGC TAC TCC AGC AAC AGT AAT GGC AAA
Arg Ser Ser Ser Lys Ala Tyr Gly Asn Gly Tyr Ser Ser Asn Ser Asn Gly Lys

                                   1107                                1134
ACA GAC TAC ATG GGG GAG GCG AGT GGA TGT CAG CTG GGG CAG GAA AAA GAA AGT
Thr Asp Tyr MET Gly Glu Ala Ser Gly Cys Gln Leu Gly Gln Glu Lys Glu Ser

                                   1161                                1188
GAA CGG CTG TGT GAG GAC CCC CCA GGC ACG GAA AGC TTT GTG AAC TGT CAA GGT
Glu Arg Leu Cys Glu Asp Pro Pro Gly Thr Glu Ser Phe Val Asn Cys Gln Gly

                                   1215                                1242
ACT GTG CCT AGC CTT AGC CTT GAT TCC CAA GGG AGG AAC TGT AGT ACA AAT GAC
Thr Val Pro Ser Leu Ser Leu Asp Ser Gln Gly Arg Asn Cys Ser Thr Asn Asp

TCA CCG CTG TAA
Ser Pro Leu End
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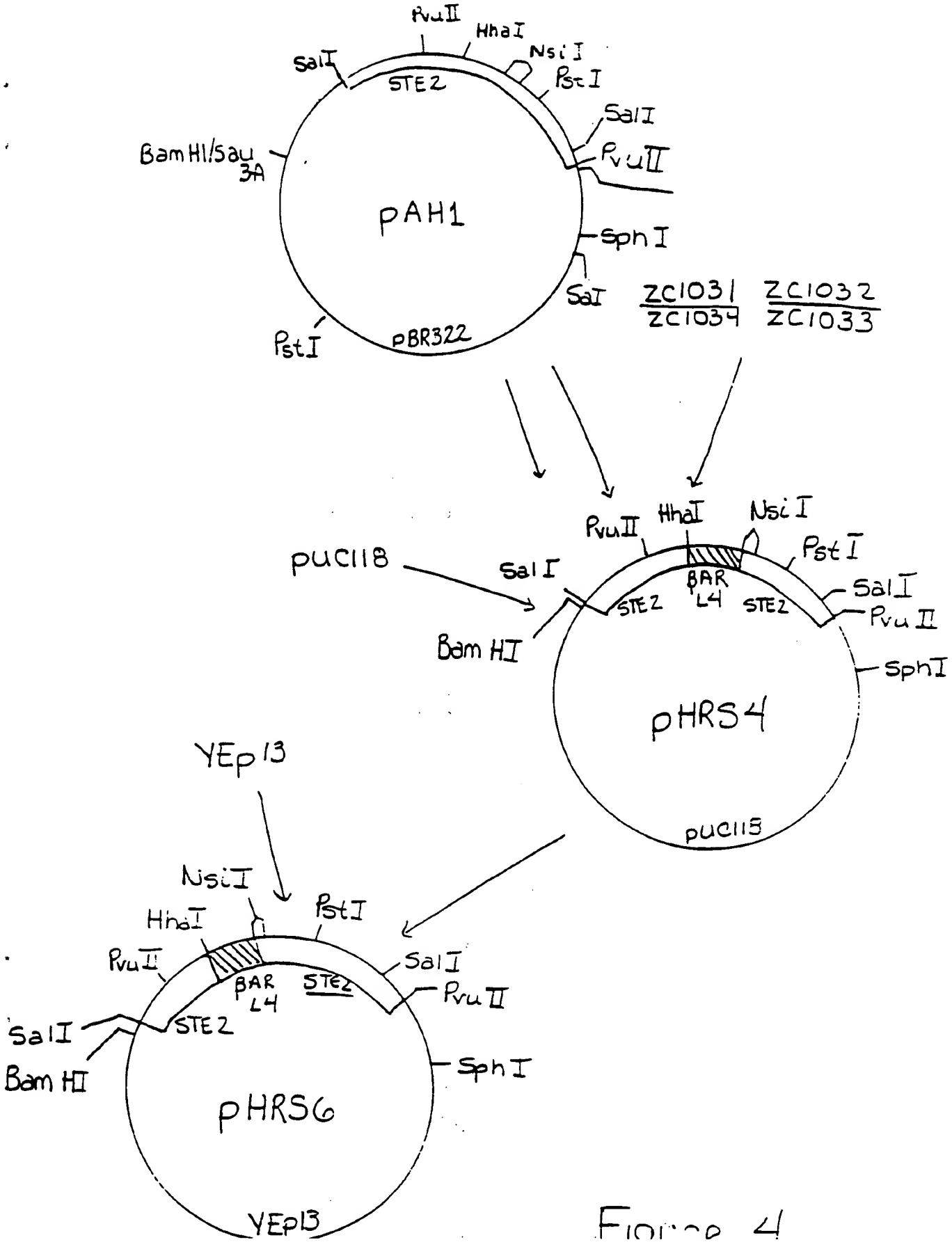
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NsiI
SalI
STE2
PstI
SalI
BamHI/Sau 3A
PvuII
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SphI
SalI
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ZC1034
ZC1032
ZC1033
PstI
pBR322
pUC118
SalI
PvuII
HhaI
NsiI
PstI
BAR
L4
STE2
STE2
SalI
PvuII
BamHI
SphI
pHRS4
pUC118
YEp13
NsiI
HhaI
PstI
PvuII
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BAR
L4
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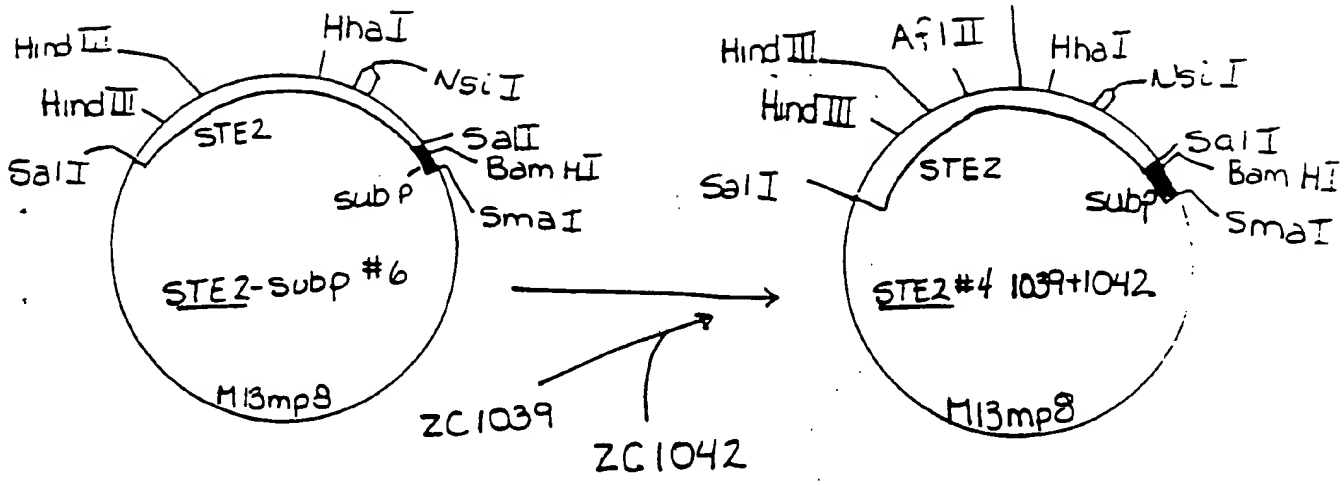
PvuII
SalI
BamHI
SphI
pHRS6
YEp13

FIGURE 4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HhaI
Hind III
NsiI
STE2
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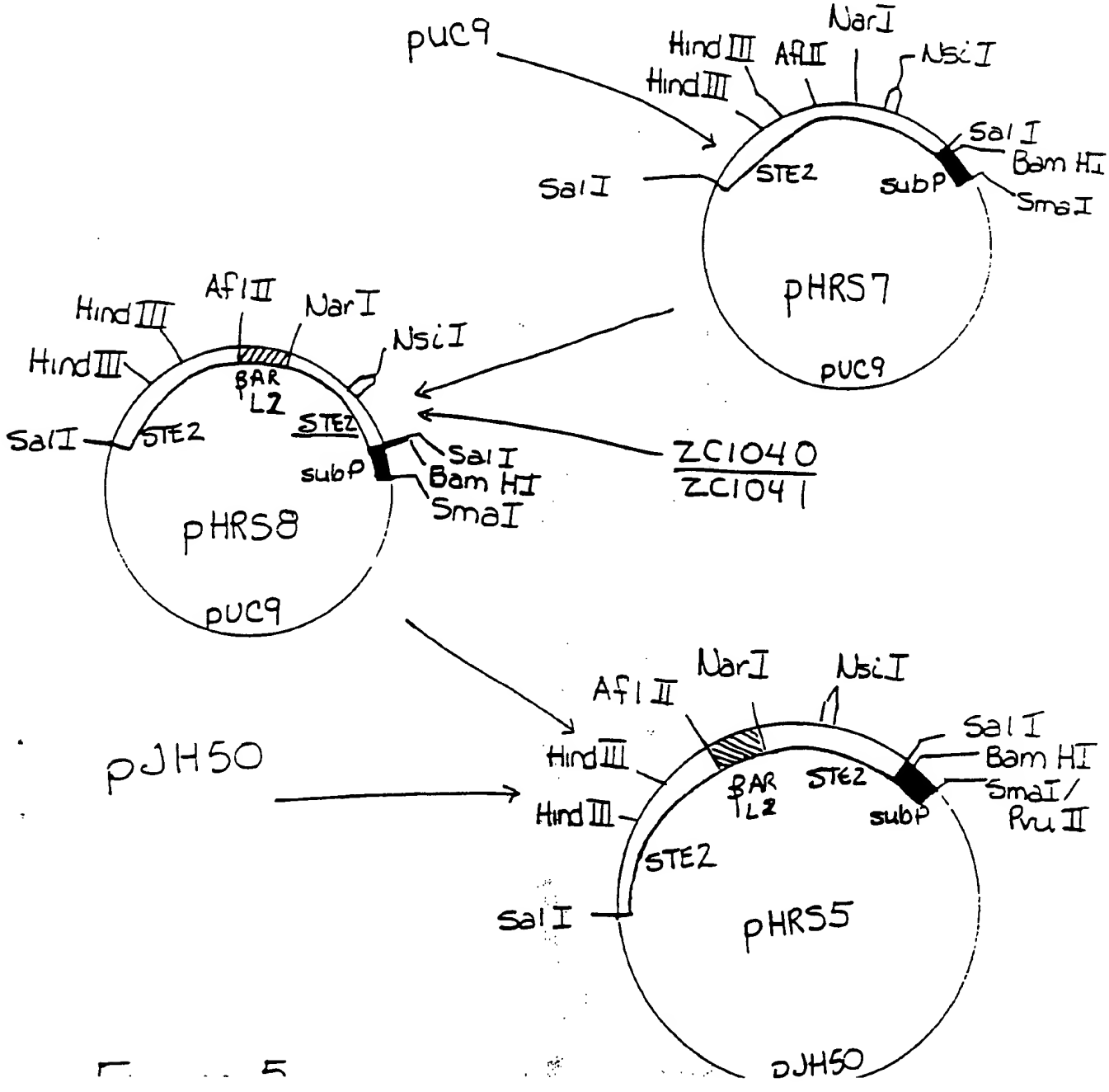
SalI
SalII
Bam HI
subP
SmaI
STE2-subP #6
M13mp8
ZC1039
ZC1042
Hind III
AflII
HhaI
Hind III
NsiI
SalI
STE2
SalI
Bam HI
subP
SmaI
STE2 #4 1039+1042
M13mp8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Hind III
SalI
Bam HI
SalI
STE2
subP
SmaI
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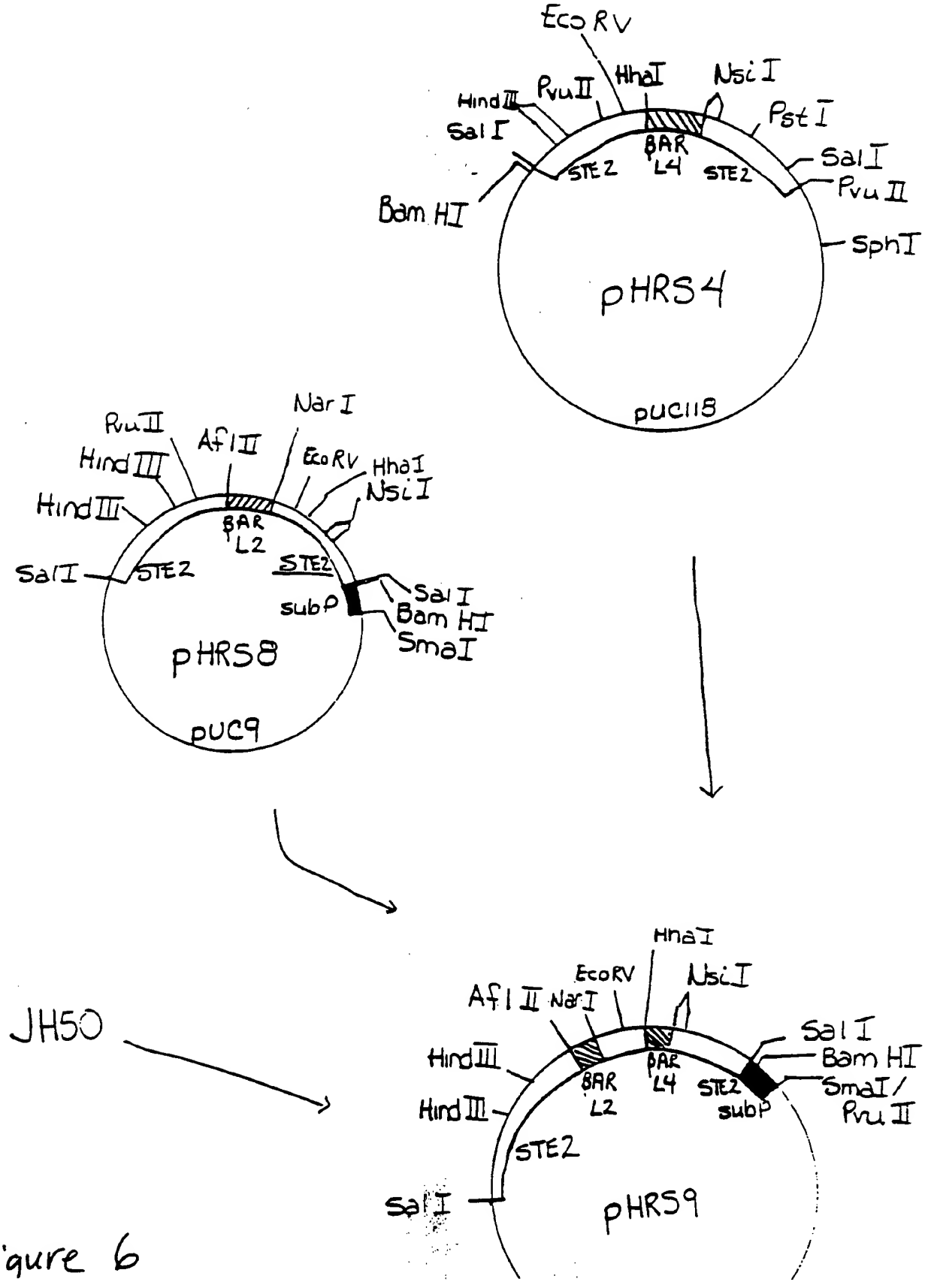
pUC9
AflII
NarI
Hind III
NsiI
Hind III
BAR
L2
SalI
STE2
STE2
SalI
Bam HI
subP
SmaI
pHRS8
pUC9
ZC1040
ZC1041
pJH50
NarI
NsiI
AflII
Hind III
SalI
Bam HI
BAR
L2
STE2
SmaI/
PvuII
subP
Hind III
STE2
SalI
pHRS5
pJH50

FIG. 5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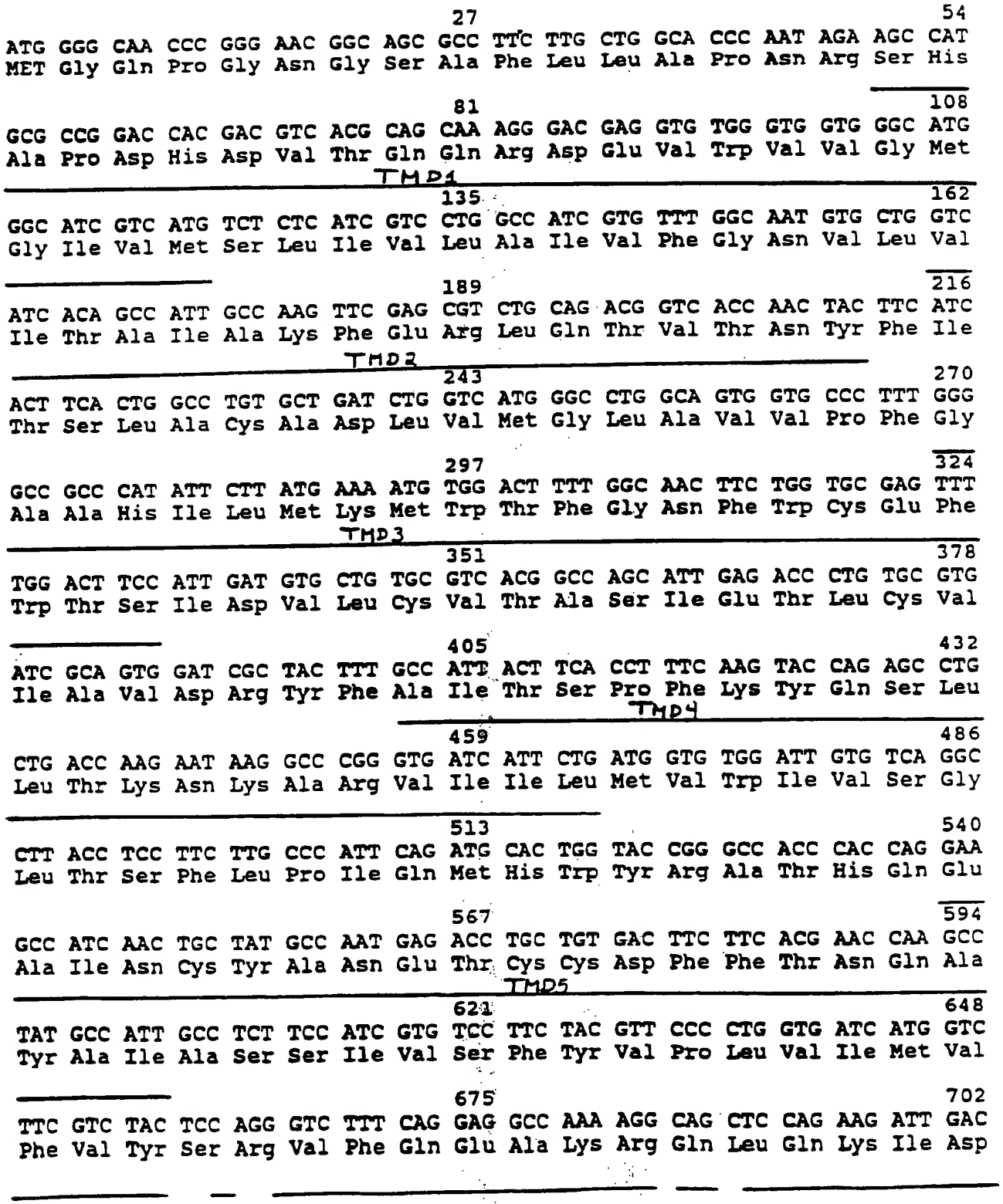
BamHI
SphI
pHRS4
pUC118
NarI
PvuII
AflII
HindIII
EcoRV
HhaI
NsiI
HindIII
BAR
L2
SalI
STE2
STE2
SalI
subP
BamHI
SmaI
pHRS8
pUC9
pJH50
HhaI
EcoRV
NsiI
AflII
NarI
SalI
HindIII
BAR
L2
BAR
L4
STE2
BamHI
HindIII
subP
SmaI/
PvuII
STE2
SalI
pHRS9

Figure 6

Figure 7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54
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MET Gly Gln Pro Gly Asn Gly Ser Ala Phe Leu Leu Ala Pro Asn Arg Ser His
81
108
GCG CCG GAC CAC GAC GTC ACG CAG CAA AGG GAC GAG GTG TGG GTG GTG GGC ATG
Ala Pro Asp His Asp Val Thr Gln Gln Arg Asp Glu Val Trp Val Val Gly Met
TMD1
135
162
GGC ATC GTC ATG TCT CTC ATC GTC CTG GCC ATC GTG TTT GGC AAT GTG CTG GTC
Gly Ile Val Met Ser Leu Ile Val Leu Ala Ile Val Phe Gly Asn Val Leu Val
189
216
ATC ACA GCC ATT GCC AAG TTC GAG CGT CTG CAG ACG GTC ACC AAC TAC TTC ATC
Ile Thr Ala Ile Ala Lys Phe Glu Arg Leu Gln Thr Val Thr Asn Tyr Phe Ile
TMD2
243
270
ACT TCA CTG GCC TGT GCT GAT CTG GTC ATG GGC CTG GCA GTG GTG CCC TTT GGG
Thr Ser Leu Ala Cys Ala Asp Leu Val Met Gly Leu Ala Val Val Pro Phe Gly
297
324
GCC GCC CAT ATT CTT ATG AAA ATG TGG ACT TTT GGC AAC TTC TGG TGC GAG TTT
Ala Ala His Ile Leu Met Lys Met Trp Thr Phe Gly Asn Phe Trp Cys Glu Phe
TMD3
351
378
TGG ACT TCC ATT GAT GTG CTG TGC GTC ACG GCC AGC ATT GAG ACC CTG TGC GTG
Trp Thr Ser Ile Asp Val Leu Cys Val Thr Ala Ser Ile Glu Thr Leu Cys Val
405
432
ATC GCA GTG GAT CGC TAC TTT GCC ATT ACT TCA CCT TTC AAG TAC CAG AGC CTG
Ile Ala Val Asp Arg Tyr Phe Ala Ile Thr Ser Pro Phe Lys Tyr Gln Ser Leu
TMD4
459
486
CTG ACC AAG AAT AAG GCC CGG GTG ATC ATT CTG ATG GTG TGG ATT GTG TCA GGC
Leu Thr Lys Asn Lys Ala Arg Val Ile Ile Leu Met Val Trp Ile Val Ser Gly
513
540
CTT ACC TCC TTC TTG CCC ATT CAG ATG CAC TGG TAC CGG GCC ACC CAC CAG GAA
Leu Thr Ser Phe Leu Pro Ile Gln Met His Trp Tyr Arg Ala Thr His Gln Glu
567
594
GCC ATC AAC TGC TAT GCC AAT GAG ACC TGC TGT GAC TTC TTC ACG AAC CAA GCC
Ala Ile Asn Cys Tyr Ala Asn Glu Thr Cys Cys Asp Phe Phe Thr Asn Gln Ala
TMD5
621
648
TAT GCC ATT GCC TCT TCC ATC GTG TCC TTC TAC GTT CCC CTG GTG ATC ATG GTC
Tyr Ala Ile Ala Ser Ser Ile Val Ser Phe Tyr Val Pro Leu Val Ile Met Val
675
702
TTC GTC TAC TCC AGG GTC TTT CAG GAG GCC AAA AGG CAG CTC CAG AAG ATT GAC
Phe Val Tyr Ser Arg Val Phe Gln Glu Ala Lys Arg Gln Leu Gln Lys Ile Asp

729 756
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Lys Ser Glu Gly Arg Phe His Val Gln Asn Leu Ser Gln Val Glu Gln Asp Gly

783 810
CGG ACG GGG CAT GGA CTC CGC AGA TCT TCC AAG TTC TGC TTG AAG GAG CAC AAA
Arg Thr Gly His Gly Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys

TMD6

837 864
GCC CTC AAG ACG TTA GGC ATC ATC ATG GGC ACT TTC ACC CTC TGC TGG CTG CCC
Ala Leu Lys Thr Leu Gly Ile Ile Met Gly Thr Phe Thr Leu Cys Trp Leu Pro

891 918
TTC TTC ATC GTT AAC ATT GTG CAT GTG ATC CAG GAT AAC CTC ATC CGT AAG GAA
Phe Phe Ile Val Asn Ile Val His Val Ile Gln Asp Asn Leu Ile Arg Lys Glu

TMD7

945 972
GTT TAC ATC CTC CTA AAT TGG ATA GGC TAT GTC AAT TCT GGT TTC AAT CCC CTT
Val Tyr Ile Leu Leu Asn Trp Ile Gly Tyr Val Asn Ser Gly Phe Asn Pro Leu

999 1026
ATC TAC TGC CGG AGC CCA GAT TTC AGG ATT GCC TTC CAG GAG CTT CTG TGC CTG
Ile Tyr Cys Arg Ser Pro Asp Phe Arg Ile Ala Phe Gln Glu Leu Leu Cys Leu

1053 1080
CGC AGG TCT TCT TTG AAG GCC TAT GGG AAT GGC TAC TCC AGC AAC GGC AAC ACA
Arg Arg Ser Ser Leu Lys Ala Tyr Gly Asn Gly Tyr Ser Ser Asn Gly Asn Thr

1107 1134
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1161 1188
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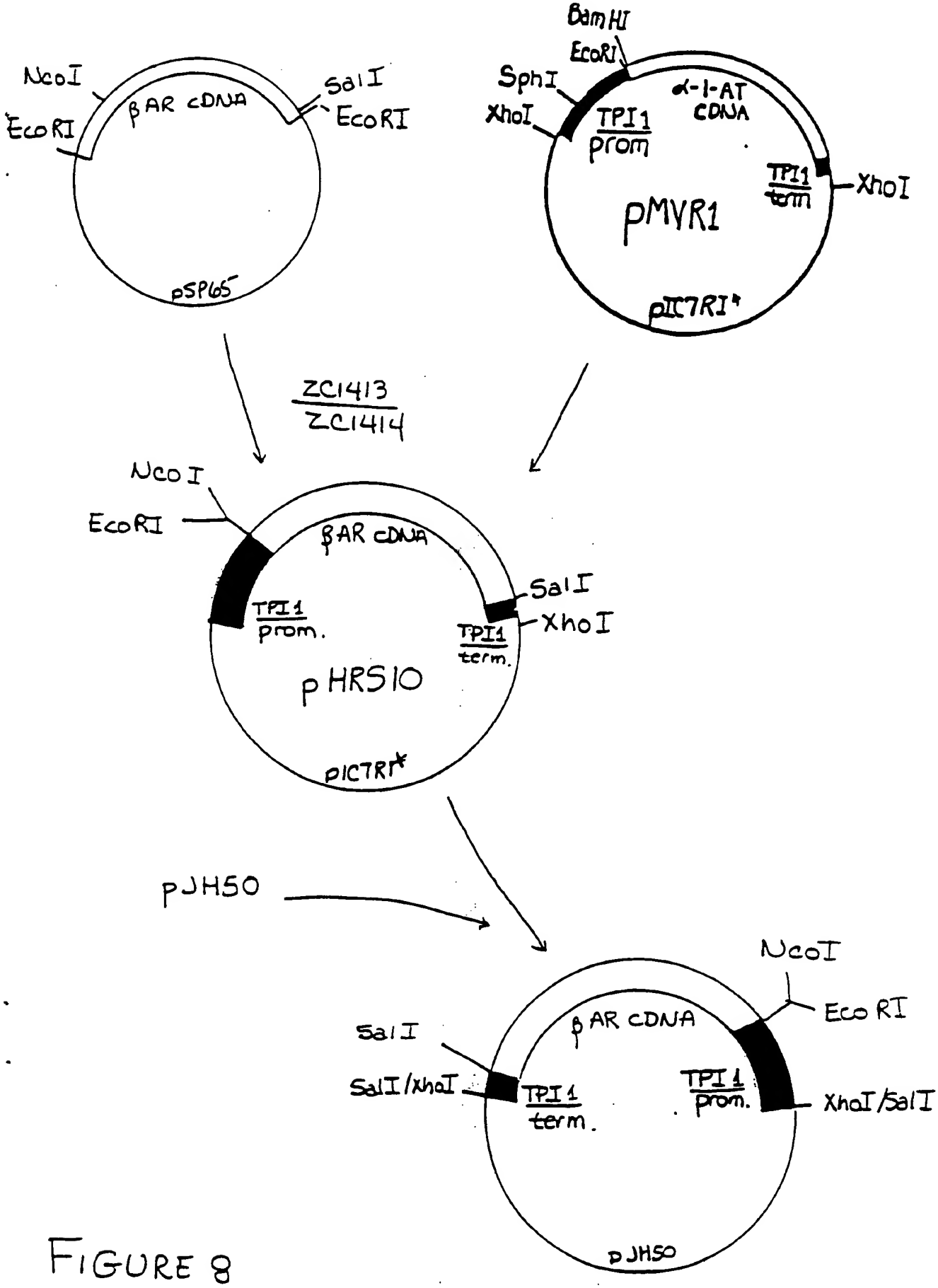
Glu Asp Leu Pro Gly Thr Glu Asp Phe Val Gly His Gln Gly Thr Val Pro Ser

1215 1242
GAT AAC ATT GAT TCA CAA GGG AGG AAT TGT AGT ACA AAT GAC TCA CTG CTG TAA
Asp Asn Ile Asp Ser Gln Gly Arg Asn Cys Ser Thr Asn Asp Ser Leu Leu End

Figure 7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TPI1 term
XhoI
pMVR1
pIC7RI*
ZC1413
ZC1414
NcoI
EcoRI
βAR cDNA
TPI1 prom.
SalI
XhoI
TPI1 term.
pHRS10
pIC7RI*
pJH50
NcoI
EcoRI
βAR cDNA
SalI
SalI/XhoI
TPI1 term.
TPI1 prom.
XhoI/SalI
pJH50

FIGURE 8

Figure 9

```
                                    27                                  54
ATG TCT GAT GCG GCT CCT TCA TTG AGC AAT CTA TTT TAT GAT CCA ACG TAT AAT
MET Ser Asp Ala Ala Pro Ser Leu Ser Asn Leu Phe Tyr Asp Pro Thr Tyr Asn

                                    81                                 108
CCT GGT CAA AGC ACC ATT AAC TAC ACT TCC ATA TAT GGG AAT GGA TCT ACC ATC
Pro Gly Gln Ser Thr Ile Asn Tyr Thr Ser Ile Tyr Gly Asn Gly Ser Thr Ile

                                   135                                 162
ACT TTC GAT GAG TTG CAA GGT TTA GTT AAC AGT ACT GTT ACT CAG GCC ATT ATG
Thr Phe Asp Glu Leu Gln Gly Leu Val Asn Ser Thr Val Thr Gln Ala Ile Met
          TMD1
                                   189                                 216
TTT GGT GTC AGA TGT GGT GCA GCT GCT TTG ACT TTG ATT GTC ATG TGG ATG ACA
Phe Gly Val Arg Cys Gly Ala Ala Ala Leu Thr Leu Ile Val Met Trp Met Thr
                                                TMD2
                                   243                                 270
TCG AGA AGC AGA AAA ACG CCG ATT TTC ATT ATC AAC CAA GTT TCA TTG TTT TTA
Ser Arg Ser Arg Lys Thr Pro Ile Phe Ile Ile Asn Gln Val Ser Leu Phe Leu

                                   297                                 324
ATC ATT TTG CAT TCT GCA CTC TAT TTT AAA TAT TTA CTG TCT AAT TAC TCT TCA
Ile Ile Leu His Ser Ala Leu Tyr Phe Lys Tyr Leu Leu Ser Asn Tyr Ser Ser

                                   351                                 378
GTG ACT TAC GCT CTC ACC GGA TTT CCT CAG TTC ATC AGT AGA GGT GAC GTT CAT
Val Thr Tyr Ala Leu Thr Gly Phe Pro Gln Phe Ile Ser Arg Gly Asp Val His
                              TMD3
                                   405                                 432
GTT TAT GGT GCT ACA AAT ATA ATT CAA GTC CTT CTT GTG GCT TCT ATT GAG ACT
Val Tyr Gly Ala Thr Asn Ile Ile Gln Val Leu Leu Val Ala Ser Ile Glu Thr

                                   459                                 486
TCA CTG GTG TTT CAG ATA AAA GTT ATT TTC ACA GGC GAC AAC TTC AAA AGG ATA
Ser Leu Val Phe Gln Ile Lys Val Ile Phe Thr Gly Asp Asn Phe Lys Arg Ile
                         TMD4
                                   513                                 540
GGT TTG ATG CTG ACG TCG ATA TCT TTC ACT TTA GGG ATT GCT ACA GTT ACC ATG
Gly Leu Met Leu Thr Ser Ile Ser Phe Thr Leu Gly Ile Ala Thr Val Thr Met

                                   567                                 594
TAT TTT GTA AGC GCT GTT AAA GGT ATG ATT GTG ACT TAT AAT GAT GTT AGT GCC
Tyr Phe Val Ser Ala Val Lys Gly Met Ile Val Thr Tyr Asn Asp Val Ser Ala
                                                TMD5
                                   621                                 648
ACC CAA GAT AAA TAC TTC AAT GCA TCC ACA ATT TTA CTT GCA TCC TCA ATA AAC
Thr Gln Asp Lys Tyr Phe Asn Ala Ser Thr Ile Leu Leu Ala Ser Ser Ile Asn

                                   675                                 702
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Phe Met Ser Phe Val Leu Val Val Lys Leu Ile Leu Ala Ile Arg Ser Arg Arg
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TMD 6

729 756
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783 810
CAA TCT TTG TTG GTT CCA TCG ATA ATA TTC ATC CTC GCA TAC AGT TTG AAA CCA
Gln Ser Leu Leu Val Pro Ser Ile Ile Phe Ile Leu Ala Tyr Ser Leu Lys Pro

TMD7

837 864
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891 918
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Leu Pro Leu Ser Ser Met Trp Ala Thr Ala Ala Asn Asn Ala Ser Lys Thr Asn

945 972
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999 1026
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Ser Ser Phe Gln Thr Asp Ser Ile Asn Asn Asp Ala Lys Ser Ser Leu Arg Ser

1053 1080
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Arg Leu Tyr Asp Leu Tyr Pro Arg Arg Lys Glu Thr Thr Ser Asp Lys His Ser

1107 1134
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Glu Arg Thr Phe Val Ser Glu Thr Ala Asp Asp Ile Glu Lys Asn Gln Phe Tyr

1161 1188
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Gln Leu Pro Thr Pro Thr Ser Ser Lys Asn Thr Arg Ile Gly Pro Phe Ala Asp

1215 1242
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1269 1296
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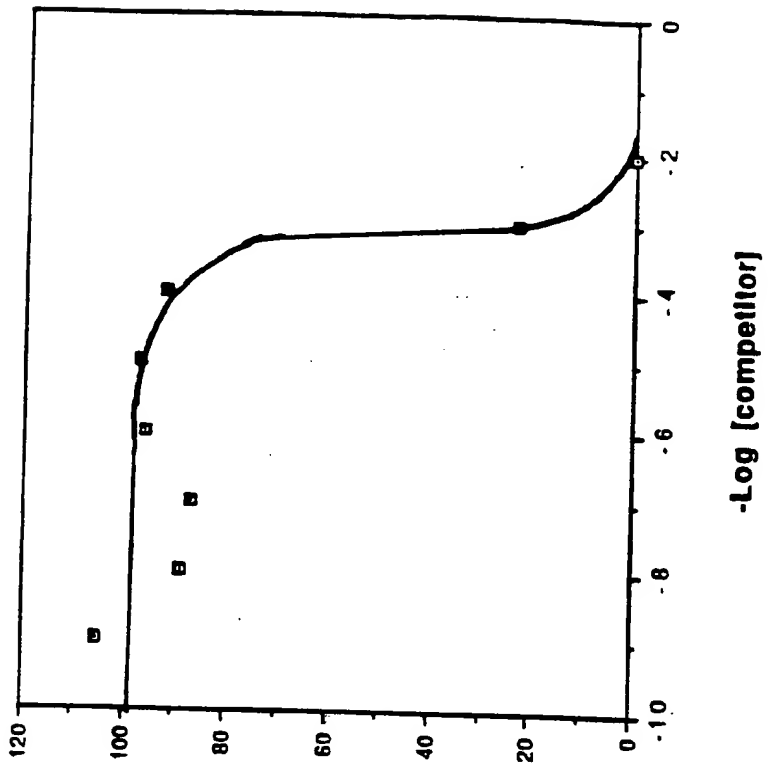
Figure 9

NOREPINEPHRINE
COMPETITION BINDING CURVE



EPINEPHRINE
COMPETITION BINDING CURVE

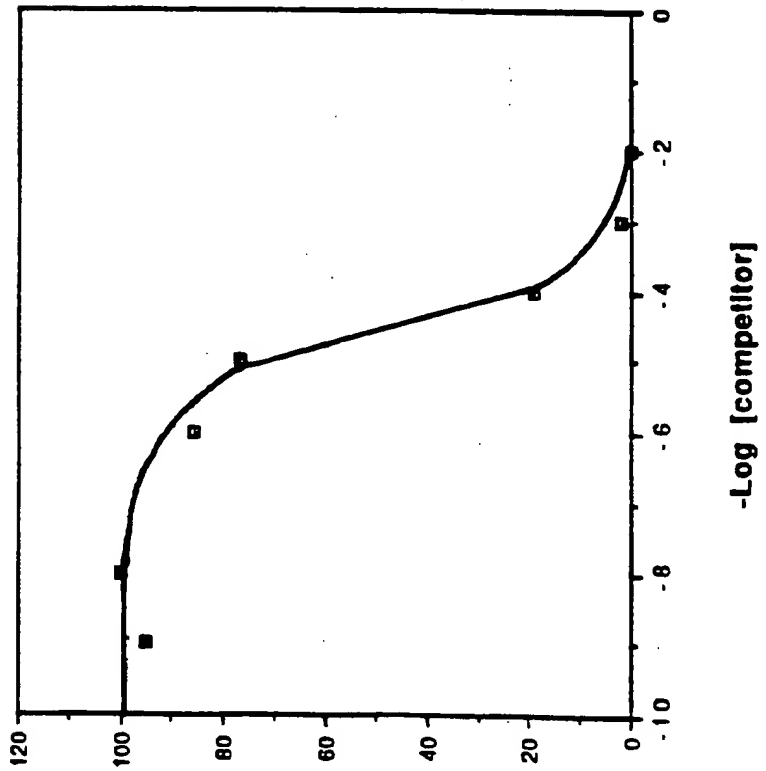


FIGURE 10

ISOPROTERENOL
COMPETITION BINDING CURVE

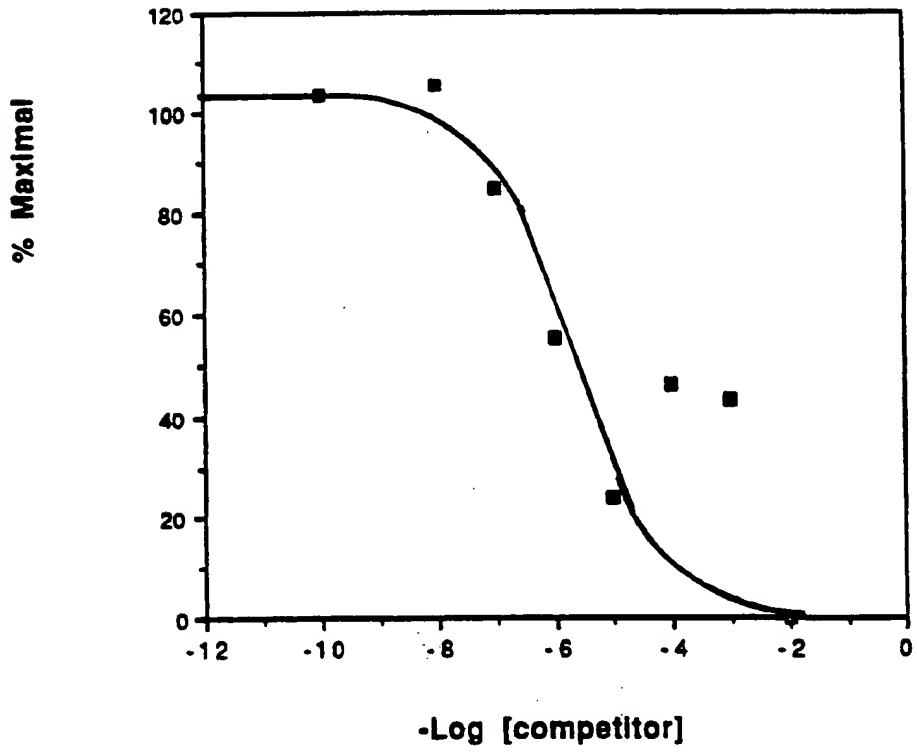


FIGURE 11